A STUDY OF THE EFFECTS OF AZADIRACHTIN ON SPECIFIC TISSUES IN THE LOCUST, *SCHISTOCERCA GREGARIA.*

by

PRIYANI ASHOKA PARANAGAMA

(B.Sc. Honours, University of Kelaniya, SRI LANKA)

Thesis submitted for the Degree of

Doctor of Philosophy

to the Faculty of Science at the

University of Glasgow

August 1994

Department of Biochemistry

University of Glasgow

Glasgow

G12 8QQ.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENT

It is with deep sense of gratitude I thank my supervisors Dr. R.H.C. Strang , Department of Biochemistry and Professor J.D. Connolly, Department of Chemistry, University of Glasgow for their tremendous encouragement, guidance and support extended to me during the course of my work. It has been a great privilege to have worked with them. Their ever-willing help and co-operation in spite of their other commitments is greatly appreciated.

My thanks are also due to Professor W. Mordue, Dr. A.J.Mordue and Dr. A.J. Nisbet for their advice and help given to me during this work.

I also express my deep appreciation to the Commission of Commonwealth Universities for financial support.

Finally I am especially grateful to my husband and my son for their unconditional support throughout.

I DEDICATE THIS THESIS TO MY FATHER

DECLARATION

I hereby declare that the thesis which follows is my own composition, that is a record of work done by myself and that it has not been presented in any previous application for a Higher Degree.

Priyani A. Paranagama.

SUMMARY

Azadirachtin was isolated from the seeds of the neem tree (*Azadirachta indica*) by solvent extraction and flash column chromatography. The recrystallised material was characterised by chromatography, NMR and melting point, and was found to have a purity of >95%. The yield, from Sri Lankan neem seeds, was 0.065% w/w. The pure compound was used to prepare the reduced derivative (22,23) dihydroazadirachtin and (22,23-$^{3}H_2$)-dihydroazadirachtin was also commercially prepared for tracer and metabolic studies. The radiolabelled tracer was used to follow tissue uptake, metabolism and excretion in the locust *Schistocerca gregaria* . It was found that an injected dose of the tracer was removed with great speed from the haemolymph, apparently by carrier-mediated specific mechanisms, into many of the locust tissues. Unlabelled analogues, injected in large excess, inhibited the clearance of the tracer to different extents and the results suggested that azadirachtin and its dihydro derivative have different affinities for the uptake mechanism. Radio-labelled dihydroazadirachtin applied topically to the locusts was shown to penetrate the insect to only a limited extent. A large fraction of the tracer was absorbed into the fat body as well as into gut, Malpighian tubules and nervous tissue. Binding of the dihydroazadirachtin was persistent and not easily displaced. There was no evidence of active excretion of dihydroazadirachtin by the Malpighian tubules. Metabolism of the dihydroazadirachtin was slow, and largely restricted to fat body and crop. Attempts, *in vivo* and *in vitro*, to show an effect of azadirachtin on Malpighian tubule function in a) water secretion and b) clearance of the sulphonic acid dye, amaranth, were unsuccessful. ^{3}H-glycine and ^{35}S-cysteine were used as tracers to examine the effects of azadirachtin on the incorporation of these amino acids into the protein and peptides in various tissues both *in vivo* and *in vitro*. The tissues examined were fat body, ovary, gut, brain and corpus cardiacum.. It was found that the terpenoid eliminated the stimulatory effects of crude neurohormonal extracts from corpus cardiacum on protein synthesis in fat body and ovary, but had no effect on basal, unstimulated synthesis. On the other hand, the azadirachtin did not inhibit the stimulation of protein synthesis in the fat body due to application of pure juvenile hormone, suggesting that the effects on protein synthesis were differential. Azadirachtin appeared to have a direct inhibitory effect on protein biosynthesis in the mid-gut, and this could partly account for observed secondary antifeedant effects. Incorporation of ^{35}S-cysteine into the polypeptides, including glutathione, synthesised in both brain and corpus cardiacum was strongly inhibited by azadirachtin. This suggested that many of the biological effects of the terpenoid are due to interference with neuroendocrine processes, most of which are mediated by peptide hormones.

CONTENTS

CHAPTER 2 EXTRACTION AND PURIFICATION OF AZADIRACHTIN AND SEVERAL OTHER COMPOUNDS FROM SEEDS OF NEEM *AZADIRACHTA INDICA.*

CHAPTER 3 UPTAKE, RETENTION, METABOLISM AND EXCRETION OF [22,23-$^3H_2$] DIHYDROAZADIRACHTIN IN *SCHISTOCERCA GREGARIA*..

LIST OF TABLES

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ILLUSTRATIONS

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 1

INTRODUCTION

1.1 Importance of biological insecticides

Many synthetic pesticides are used for protection of growing crops and stored products. They have resulted in environmental problems and disturbances of the local ecology due to increasing pest resistance and destruction of non-target beneficial insects. The problems associated with the large-scale use of synthetic insecticides have dictated the need for effective, biodegradable pest control materials with greater selectivity. Because insect damage of plants results from feeding or from transmission of plant pathogens during feeding, chemicals which reduce pest injury by rendering plants unattractives or unpalatable can be considered as potential substitutes for insecticides (Saxena et al., 1981).

The "first generation" of insecticides was introduced about 100 years ago. They were natural plant insecticides such as pyrethrins, nicotine, rotenone or ryanodine. Generally, the efficiency and the selectivity of these products against insects was somewhat limited, but they were the best compounds available until about 1940, by which time some insect populations had already developed resistance against some of these compounds. Around 1940 the synthetic insecticides, such as DDT, were introduced as the "second generation" of insecticides (Forgash, 1984). They were very efficient, neuro active compounds, e.g. the chlorinated hydrocarbons, the organophosphates, the carbamates and the pyrethroids. These developments had a profound impact on agriculture and public health. Productivity increased substantially and diseases such as malaria were eradicated from entire geographic areas. These groups of highly potent neurotoxins dominated the insecticide market. Initially, high potency and broad spectrum activity were most important. Later, other aspects such as reduced toxicity against vertebrate and entomophageous insects or reduced environmental stability became desirable. After the achievement of potency and efficiency during the period 1940-1960, research began to focus more on factors such as selectivity and environmental safety. In the late 1960's a "third generation" of

insecticides was born, insect growth regulators. Compounds disturbing hormone signalling or chitin synthesis, such as juvenile hormone analogues, ecdysones, precocenes and acylureas are generally selective and environmentally safe. However, they act slowly, often allowing the insects to consume considerable quantities of their host plants before they die. Then another group of compounds, called the "fourth generation" of insecticides, and including antifeedants and pheromones were introduced. They are potent, broad spectrum antifeedants often with systemic activity (Eder, et al. 1985).

The chemicals, which when perceived, reduce or prevent insect feeding, and consequently, growth, development, survival and reproduction, are important defence barriers in some plants against insect attack. They are generally pest-specific and therefore, harmless to non-target organisms. Research on the biological activity and chemistry of antifeedants has been emphasized, because of their biodegradable nature and relative safety to beneficial organisms in the environment (Norris, 1986). Hence in recent years, there is increased dependence on the use of plant products for insect pest management in crop plants.

The plants that have shown potential in pest control during the past few years are; neem, *Azadirachta indica*; Chinaberry, *Melia toosenden* L. and *M. azedarach* L.; West indian mahogany, *Swietenia mahagoni* Jacq; custard apple, *Annona squamosa* L.; African marigold, *Tagetes erecta* L.; French marigold, *Tagetes patula* L.; and thunder gold-vine, *Tripterygium wilfordii* Hook. Today, however, only a few active ingredients produced by various natural organisms are commercial pesticides (Table 1.1).

Table 1.1 Natural compounds as commercial pesticides in world markets (Naumann, 1994)

Natural product	Origin	Application e.g.
Pyrethrum	plant	garden pests
Avermectin	bacterial	spider mites
Microbial antibiotics	bacterial fungi	rice fungal pests
Rotenone	plant	garden pests
Azadirachtin	plant	garden pests, fungal pests agricultural pests etc.
Nicotine	plant	green house

Officially allowed crude extracts of natural products for plant protection in organic farming are: sea weeds, Pyrethrum, Derris roots, *Quassia* wood extract, *Ryania* wood dust, *Bacillus thuringiensis* endotoxin, soft soap, plant oils, *Reynoutria* extracts, wild herb extracts, Neem seed extracts . Among these plant products, the neem extracts, originating from the neem tree, *Azadirachta indica* , are available in many parts of the tropical and subtropical regions of the world.The neem extracts, containing azadirachtin* (Fig.1.2) are of particular interest at present, because there seems to be a realistic basis for providing many tons of active ingredients for world-wide protection purposes. At present the use of such extracts is largely restricted to local farmers in semiarid, tropical areas around the globe where the tree is growing.

*It has been discovered the major active ingredient in the neem extracts is azadirachtin, which is a tetranortriterpenoid (see detail in section 1.8).

The extracts of the seed of several million trees would be needed to protect an area of 10^6 ha of a normal agricultural crop. One neem tree may provide the azadirachtin needed to protect 0.3-1 ha, taking an application rate of 20-50g/ha against, for example, lepidopterous larvae. However, the sensitivity of various species to azadirachtin varies widely. In the meantime, this botanical source has been registered in several countries, including the USA, for food crops (Naumann, 1994).

In developing countries the use of neem seed extracts, neem oil, and neem cake against vegetable and fruit-tree pests is feasible even at the level of the peasant farmer. On the other hand, in industrialized countries and India, some alcoholic formulated neem seed extracts are being developed. Although their efficacy is sometimes inferior to that of toxic, broad-spectrum, synthetic insecticides, the degree of reduction of pest populations is, as a rule, quite sufficient (Ermel, et al., 1987). A comparison of the efficacy of neem extract with synthetic pesticides against *Plutella xylostella* has been carried out (Table 1.2).

In these trials aqueous and enriched alcoholic seed kernel extracts (AZT-VR-K) alone or in combination with the *Bacillus thuringiensis*- based product, Thuricide, were compared to each other and to the synthetic insecticides a "Selecron" (profenofos) and BAY SIR 14591 (IGR, developed by Bayer AG). Cabbage plants were treated five times starting eleven days after transplanting. The order of effectiveness with reference to the yield was BAY SIR 14591 > AZT-VR-K (0.2%) > AZT-VR-K (0.2%) + Thuricide > aqueous kernel extract (25g/l) > aqueous kernel extract (25g/l) + Thuricide > Selecron > Thuricide. The development of the larvae in the neem treated plots was seriously affected so that considerably lower numbers of last instar larvae, prepupae, and pupae were found. Due to heavy infestation pressure the feeding activity of larvae could not be prevented completely in the neem plots, but most of the cabbage heads showed only light, tolerable damage (Kirsch, 1987).

Table 1.2 Effect of neem extract and conventional insecticide treatments on cabbage yield and head damage (Kirsch, 1987).

Insecticide, conc. or applied amount	Yield (tons/ha)	Head damage*
Control	34.6	4.9
Selectron 500 EC, 0.05%	42.3	4.9
BAY SIR 14591 250 EC, 50g/ha	71.1	1.0
Thuricide HP, 1kg/ha	34.0	4.8
AZT-VR-K EC, 0.20%	52.9	2.6
AZT-VR-K EC, 0.20% + Thuricide HP 1kg/ha	47.3	2.3
ANSKE, 25g NSK/l	45.5	1.5
ANSKE, 25g NSK/l + Thuridice HP HP 1kg/ha	44.3	1.4

* Damage rating: 1 = heads with no damage; 2 = heads with light damage, no trimming required; 3 = heads with light damage, trimming of one leaf required; 4 = heads with moderate damage, trimming of two to three leaves required; 5 = heads with severe damage, trimming of more than three leaves required.

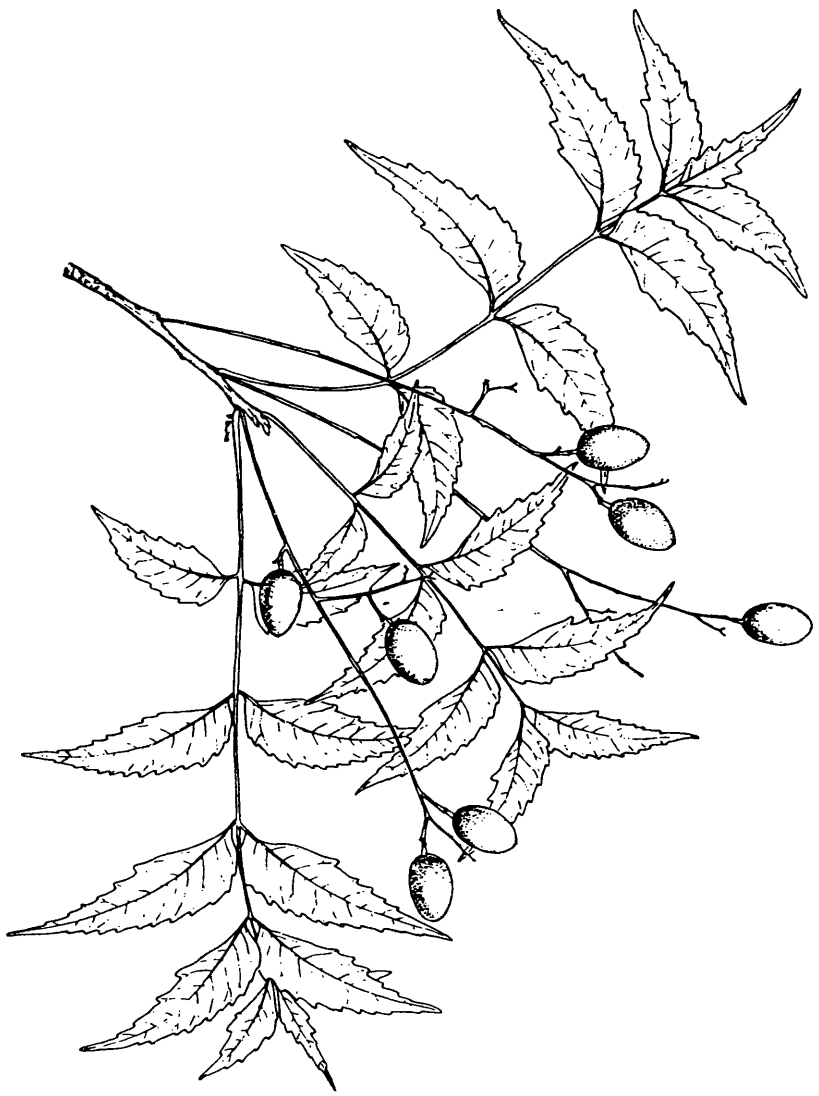
Therefore extracts of the neem tree are of great potential importance due to the fact that they have low mammalian toxicity and low environmental problems in contrast to the hazards of toxic synthetic pesticides. The interest in neem seed kernel extracts is increasing in the developing countries in the hope that these may be used to solve the problems caused by synthetic pesticides.

1.2 Neem tree

Neem, *Azadirachta indica* A. Juss (Fig. 1.1), is a member of the Meliaceae family. The trees are attractive broad-leaved evergreens that can grow up to 30m tall and 2.5m in girth. Their spreading branches form rounded crowns as much as 10m across. They remain in leaf except during extreme drought, when the leaves may fall off. The short, usually straight trunk has a moderately thick, strongly furrowed bark. The roots penetrate the soil deeply, where the site permits, and particularly when injured, they produce suckers. The small, white, bisexual flowers are borne in axillary clusters. They have a honey like scent and attract many bees. Neem honey is popular, and reportedly contains no azadirachtin. The fruit is a smooth, ellipsoidal drupe, up to almost 2 cm long. When ripe, it is yellow or greenish yellow, and comprises a sweet pulp enclosing a seed. The seed is composed of a shell and a kernel (sometimes two or three kernels), each about the same weight. It is the kernel that is used most in pest control. A neem tree normally begins bearing fruit after 3-5 years, becomes fully producing in 10 years, and from then on can produce up to 50 kg of fruit annually. It may live more than two centuries (Ruskin, 1992).

The exact origin of the neem tree is uncertain. Some say it is native to the whole Indian subcontinent, and others attribute it to dry forest areas throughout all of South and Southeast Asia, including Pakistan, Sri Lanka, Thailand, Malaysia, and Indonesia. The tree is easily propagated, both sexually and vegetatively. It can be planted using seeds, seedlings, saplings, root suckers, or tissue culture. However, it is normally grown from seed, either planted directly on the site or transplanted as seedlings from a nursery (Ruskin, 1992). Almost every part of the neem tree, including its roots, leaves and fruits offers great potential for agriculture, industrial and commercial exploitation.

Fig. 1.1 A twig of neem showing characteristic leaves and fruits.



For instance, the seed oil has commercial possibilities for lighting and heating and in the manufacture of wax, lubricants and soap. The low cost of preparing neem extracts for insecticidal use contrasts with that of synthetic pesticides and is really a major factor behind its wide use in developing countries (Randhawa et al., 1993).

1.3. The use of neem in Sri Lanka

Neem is an important plant, which has multiple uses for farmers and others. It is called 'Kohomba' in Sinhalese and 'Vempu' in Tamil and is common in the dry zone of Sri Lanka. It grows in the wild, but it is also cultivated by the people because of its manifold uses.

Annually more than 700,000 Kg of pesticides, herbicides and fungicides are used in Sri Lanka in agricultural to control pests, weeds, herbs and fungi. The neem tree in Sri Lanka could be utilized by small local farmers who cannot afford to buy the expensive synthetic pesticides. Most of them lack adequate knowledge of the handling of hazardous insecticides.It was found that 74% of the admissions to the hospitals as a result of poisoning are due to pesticide poisoning (Wimalasena, S., personal communication). For these farmers, the neem tree is a good botanical source for crop pest management, and the use of neem water extracts are safe to handle.

Neem leaves are used as an insect repellent in storage of rice in containers. The leaves are chopped and kept over the heap of rice in the container. The container is also covered with leaves. The odour emitted by the leaves drives away the insect pests. As the green leaves dry up, they are replaced periodically. Green leaves are also used by vendors who sell fast food, to drive away insects, especially flies. They either hold the leaves in a bundle in their hand, or hang them over the food. Green leaves are used as pesticide- manure. Green leaves are also used as fodder for cattle, and appear to combat worm infection in livestock. Neem is also an anthelmintic for cattle. The milk obtained after feeding this leaf to milch-cows is bitter, but is supposed to be good for

the health of human beings. The dry leaves are also burnt in front of homes and the fumes drive away mosquitoes and other insects.

The seed is collected, sun-dried, and stored. They can then be burnt with paddy husk in clay pots to repel mosquitoes in houses. Seed powder is mixed with cereal grains in storage to control the insect pests of stored products. Seed paste is used as a detergent (shampoo) for removing lice from the head. Seed oil is obtained by 'milling' the dry seeds. The oil is used as a repellent against insect pests in houses and cattle sheds. The oil is smeared on wooden surfaces in houses to repel insect pests, and is also used in the form of a fumigant. In native medical practice, neem oil is used as an anthelmintic in man and livestock ; and also against chronic forms of skin diseases, stomach ulcers and rheumatism (Fernando, 1982). It is a parasiticide for ringworm, scabies and other skin diseases. It destroys parasites even if they are present deep in the skin. In malarial fever this oil is effective when given internally periodically at intervals of one or two days. The oil is also a powerful and effective antiseptic. Neem oil is used to manufacture soap, the use of which safeguards the skin from microbial infection. This soap is available in the shops and is preferred to other soaps because of its medicinal value.

It is believed that the dried flowers can be used in preparing an effective food for diabetics called 'Vedakam' which is sold in local shops. It consists entirely of dried neem flowers mixed with black gram (chick pea) paste. Neem 'honey' is obtained from neem flowers. It is used by village people, especially the children, to ensure good health, and to prevent infection.

Neem timber is of great value, durable, and preferred over other species for decorative work and carving. It is used for making doors and windows because there is no danger of this timber being attacked by termites due to its antifeedant affect. The bark taken from the living tree is boiled in water and this decoction is used as a tonic to relieve muscular pain in influenza. The bark is astringent and bitter. The aqueous

extract is used as a febrifuge and drinking this and tonic in local medicine. However, it has been noted that, following removal of the bark, timber of inferior quality is produced. The bark of the root of neem is effective in relieving pain. The thin twigs are traditionally used for cleaning the teeth and ensure strength and firmness of both teeth and gums (Trimen, 1974).

1.4. Neem extracts as biological insecticides.

The cost of preparing neem extracts for insecticidal use contrasts with that of synthetic pesticides. It has been estimated (Redknap, 1981) that neem applications to repel insects can be as low as one tenth of the cost of malathion. This is a major factor behind its wide use in developing countries.

The most important neem products used in numerous trials are as follows: neem seed oil and neem seed cake; aqueous neem seed kernel and leaf extracts ("suspensions"); alcoholic (ethanolic, methanolic) seed kernel and leaf extracts; enriched, formulated seed kernel extracts.

Neem seed oil is pressed from neem seeds in the cold by using oil presses or extraction with alcohols or other solvents using a Soxhlet apparatus. Small amounts of oil can be obtained by kneading neem seed powder by hand after adding some water (Dreyer, 1987). Neem kernels contain up to 50% of oil. Neem oil is emulsified by addition of emulsifiers. It may be sprayed after mixing with water at concentrations of a few percent (3 to 5%) or at higher concentrations, which may, however, lead to phytotoxicity in some plant species.

Neem seed cake is the residue of seed kernels after the oil has been removed. This cake is a useful organic fertilizer, containing several percent of nitrogen as well as some azadirachtin and/ or other active principles. It also has nitrification properties. This cake, worked into the soil in various concentrations, has proved to be effective against some soil and root-infesting nematodes. Fresh and dried neem leaves are also

somewhat effective against nematodes after they have been worked into the soil, although considerable amounts are needed to obtain satisfactory results.

Aqueous extracts ("suspensions") are prepared from ground or pounded dried neem seeds or leaves. The former are often preferred as they are more active. Usually about 25 to 50g of seed kernels are used per litre of water. If unshelled seeds are used, double the quantity is needed. The extraction process lasts 5 to 6 hours. The larger particles are removed by filtration through a piece of cloth and the spray is ready for application at a high volume (Dreyer, 1987).

One-step alcoholic seed kernel or leaf extracts have also been used, but their production is more expensive and also more time-consuming than that of water extracts. Alcoholic extracts can be obtained by extraction of ground or pounded seed kernels or leaves in the cold or by means of Soxhlet apparatus. For successful pest control, concentrations of a few percent (1 to 2% for seed extract, 2 to 4% for leaf extracts) are needed (Adhikery, 1983; Steets, 1976; Karel, 1987).

Enriched, formulated seed kernel products are either based on alcoholic extracts, which are purified in some steps to increase their azadirachtin content, as in the case of U.S. patented "Margosan-O" (Larson, 1987), or extracts obtained with an azeotropic mixture of methanol and methyl tertiary-butyl ether, also purified in some steps, as in the case of AZT-VR-K (Feuerhake, 1984, Feuerhake et al.,1985).

There are several active compounds in neem seeds, but the dominant substance is the tetranortriterpenoid, azadirachtin. Normally the best results in controlling insect pests are obtained with seed extracts containing the highest amounts of this compound. For this reason it is of great importance to collect seeds of good quality and to dry and store them properly to prevent losses of the active ingredients and contamination by fungi especially under humid conditions.

The first commercial product of neem, "Margosan-O" (W.R. Grace & Co., Cambridge, MA, USA) was registered in the USA in 1985. Several commercial and

semicommercial preparations are now available (Table 1.3). Neem seed oil is often a starting material for such insecticides and its biological activity is closely related to its azadirachtin content (Isman et al., 1990). However, with the different formulations of neem used, and the many varied modes of application, detailed comparisons of efficacy against different pest species are extremely difficult to make. For example, limonoid mixtures may be more effective than azadirachtin alone; neem oil itself has insecticidal properties unrelated to its azadirachtin content and crude formulations contain volatile repellent components.

1.5. Use against pests

The neem tree has emerged as a most important source of insecticides. All parts of the tree are biologically active. The maximal insecticidal activity, however, is in the seed kernel. The kernel extracts and pure compounds isolated from the seed have shown diverse biological effects against insects. These include repellent, feeding and oviposition deterrent and growth regulatory and sterilizing effects. In addition, neem is also reported to have direct toxicity and to impair egg viability.

The number of insects tested with neem has now increased to more than 300 species (Singh, 1993). Most of the above evaluations deal with pests of agricultural importance and include almost all the key pests of agriculture. Some active ingredients of the seeds and leaves of the tropical neem tree, especially the tetranortriterpenoid azadirachtin, influence the feeding behaviour, metamorphosis, fecundity, and fitness of numerous insect species belonging to various orders. Some spider mites are also affected. Apart from some smaller, less important groups in other insect orders, Saltatoria, Homoptera, Heteroptera, Coleoptera, Lepidoptera, Hymenoptera, and Diptera have proved to be sensitive, at least in laboratory experiments.

Table 1.3- Commercially produced neem-based pesticidal products (Parmar et al., 1993).

Commercial Product	Active ingredient(s) or source	Activity claimed
Margosan-O	Azadirachtin	Insecticide, miticide
Meen	Azadirachtin	Growth reg., repellent, antifeedant
Azitin-EC	Azadirachtin	Insecticide/miticide
Neemazal	Azadirachtin	Inseticide
Godrej Achook	Azadirachtin, azadiradione, nimbocinol, epinimbocinol	Antifeedant, repellant/ deterrent & growth reg.
Field Marshal	Azadirachtin	Antifeedant, repellant
Margocide-CK	Azadirachtin	Antifeedant, growth reg., ovicidal, nematicidal
Moskit	oil	Mosquit repellant
Neem based EC	Kernel or oil	Pesticidal
Neem oil emulsion	Oil	Pesticidal
Neemgold	Azadirachtin, kernel extract	Antifeedant
Neemrich	Extracts	Warehouse pests & antifeedant
Nimba	Kernel based powder	Pesticidal
Wellgro	Neem kernel Powder	Repellent, fungus inhibitory, antiviral, & plant nutrition

1.5.1. Phytophagous insects

Neem products can control effectively a considerable number of vegetable pests, fruit-tree pests, and pests of ornamental crops including trees, shrubs, and flowers. The best results have been obtained by application of extracts from neem seeds with alcohols (methanol, ethanol). Extracts from neem leaves are generally less effective since greater amounts of raw material have to be used to obtain results comparable to those of the seed extracts (Hongo et al., 1986). Neem oil also showed a lower degree of efficacy than whole seed extracts. However, the effect of neem products varied with the pest species tested and the progeny of the materials used. This is not surprising in view of the problems of standardization.

Lepidopterous larvae have been the main target pests of neem application (Rossner et al., 1987). They are the most important group of harmful insects in warm climates. In most field experiments against caterpillars, satisfactory to very good results were obtained, especially by application of seed kernel extracts. The feeding activity and metamorphosis of the insects were seriously disturbed by the active ingredients of these extracts, leading to a decline of pest populations which was often equal to the effect of a number of well-known synthetic pesticides. In some cases the effects of neem products were better than those of synthetic products, especially in control of the diamondback moth, *Plutella xylostella.* The latter develops resistance to all major groups of pesticides rather quickly, especially in Asia, but also to microbial pesticides based on *Bacillus thuringiensis* (Schmutterer, 1985). Interestingly, a selection for resistance of *P. xylostella* against neem products during forty generations in the laboratory was not successful, giving hope that resistance to neem compounds may not rapidly appear (Jacobson, 1988).

Cabbage field plots heavily infested with the diamondback moth, *Plutella xylostella,* were treated with different neem products at intervals of seven days starting five weeks after transplanting (Adhikary, 1983). A water extract of powdered neem

leaves (suspension) (40g/l) gave no protection relative to the control. On the other hand, a neem leaf methanolic extract at 4% significantly increased the yield and reduced the number of damaged heads and of larvae per infested head. The best results were obtained with methanolic extract of neem kernels. At both concentrations tested (2 and 4%) yields were considerably higher than in the control. There was no significant difference between the plots sprayed with the synthetic insecticides "Mevinphos" (0.05%) or "Deltamethrin" (0.02%) and those sprayed with the neem kernel extracts.

Sprays based on 25 and 50g of neem powder per litre of water prevented any feeding damage by the cabbage moth and also controlled of the cabbage head webworm, *Hellula undalis*. Consequently, the number of the cabbage heads harvested, the quality, and the weight per head were considerably higher in the neem plots. "Dipel", a microbiological product based on *Bacillus thuringiensis*, proved to be considerably less active than the neem extracts (Dreyer, 1987).

In field tests against the cabbage webworm, *Crocidolomia binotalis,* finely ground or oven-dried leaves of neem were stirred for about 24h in 95% ethanol. From the dried crude neem leaf extract a 2% solution was prepared which was applied weekly to cabbage, starting from the third week after transplantation of seedlings. The neem treatment reduced the number of infested plants by about 40% in comparison with the control. Egg-laying by adult moths was reduced by about 60%. Methanolic extracts of neem leaves gave higher yields and were more effective than ethanolic and aqueous extracts and therefore may also give slightly better results in the field (Fagoonee, 1979).

In a field test in India, the efficacy of different neem products was evaluated against insect pests of radish. An aqueous seed kernel extract, ethanolic seed kernel extract, and neem oil, all at concentrations of 1, 2, and 3%, respectively, were sprayed on young radish plants seven, seventeen, twenty seven, and thirty seven days after sowing. All neem treatments reduced leaf damage by the flea beetle, *Phyllotreta*

downsei. The best results were obtained with an aqueous seed kernel extract, which gave between 60 and 68% protection, followed by ethanolic extract and neem oil (Abdul Kareem, 1981).

A weekly application of ripe neem fruits at 112.5g/l and a fortnightly application of a neem fruit/water suspension (85g/l) controlled efficiently the African melon ladybird, *Henosepilachna elaterii* (*Epliachna chrysomelina*). The number of cucumber per plant was higher than in the malathion treatment (85 ml / 4.5l) (Redknap, 1981).

Laboratory tests showed that neem kernel extracts with nonpolar solvents are more effective than with polar ones in the control of the carmine spider mite, *Tetranychus cinnabarinus*, a pest of beans and other vegetables. The best results were obtained with pentane, followed by chloroform, n-butanol, acetone, methanol, and water. Adult females were repelled from treated bean leaves and laid fewer eggs. However, the chloroform and ethanol extracts caused phytotoxic effects. Adult females of the mite, when sprayed directly with a pentane or an acetone extract, showed reduced fecundity and high mortality (Mansour et al., 1984).

The effectiveness of neem extracts in controlling several bean pests was compared to that of other plant extracts (tomato, hot pepper) and the synthetic insecticide "Lindane". Aqueous extracts of neem seed kernels at 2% and of neem leaves at 4%, sprayed in the field on bean plants, halved the incidence and the damage of the flower thrips, *Taeniothrips sjostedti*. In the neem treatments the number of larvae of the pod borers, *Maruca restulalis* and *Heliothis armigera*, and the pod sucking bug, *Acanthomia horrida,* were also reduced (by up to 40%). The seed extracts proved to be more active than the leaf extract. The results of the "Lindane" plots were not significantly different from those of the neem plots (Hongo et al., 1986).

Application of aqueous neem seed kernel extract ("suspension") in India at 1, 5, and 8% on pigeon pea, *Cajanus cajan*, controlled efficiently the pod fly,

Melanagromyza obtusa, and the pod borer, *Heliothis armigera* (Jain et al., 1986; Srivastava et al., 1984). The efficacy was comparable to that of synthetic pesticides. Similar results were obtained with neem oil and ethanolic extracts of seeds against *M. obtusa, H. armigera,* and *Maruca testulalis* on the same crop (Parmer, 1987).

More recent work relates to new applications of azadirachtin in the management of pests of ornamental crops (Price et al., 1990). Both contact and systemic action of the neem-based insecticide "Margosan-O" on the spiny bollworm, *Earias insulana* and the leafhopper, *Assymmetrasca decedens* were measured (Meisner et al., 1990, 1992). Insect growth regulatory (IGR) effects of low topical doses of "Margosan-O" to *Spodoptera littoralis* larvae (Meisner et al., 1992), and varying susceptibility of nymphal instars of *Aphis fabae* to "Margosan-O" were found (Dimetry et al., 1992). For field applications, careful consideration is required of the stability of a compound in the particular climatic conditions. Necessary steps for its stabilization should be taken. For example, the antifeedant potency of azadirachtin exposed to sunlight for seven days was reduced by more than half compared with non-exposed azadirachtin against *S. frugiperda* first instars. No activity remained after sixteen days (Stokes et al., 1982).

The effect of azadirachtin and salannin (Fig. 1.2) on the striped cucumber beetle, *Acalymma vittatum* and the spotted cucumber beetle, *Diabrotica undecimpunctata* , was tested in laboratory and greenhouse trials in the U.S. Leaf disks, dipped in an azadirachtin solution of 0.01%, stopped feeding by *D. undecimpunctata* by 98%. Salannin was also very active against the former but less active against the latter. Further greenhouse trials showed that azadirachtin concentrations of 0.1% gave good protection against *A. vittatum* for three days. To extend the effect for a longer period and to reduce transmission of diseases by the beetles, concentrations of at least 0.5% had to be applied. In greenhouse tests it was

demonstrated that azadirachtin can act systemically when the roots of plants are immersed in azadirachtin solutions (Reed et al., 1982)

1.5.2. Stored product pests

Neem has proved to be effective in protecting stored products, particularly grain, whose losses, if untreated, can be high. Such losses are frequent in developing countries due to the inability to apply expensive chemical pesticides. Effects on stored product pests include antifeedancy, oviposition, reduced egg hatch and emergence, and direct lethality (Morallo-Rejesus et al., 1990; Ivbijaro, 1990; Naquvi et al., 1990). The protection of neem may persist for a number of months, although this varies among insect species, and is perhaps related to the behaviour of the insects (Makanjuola, 1989). A clear benefit of neem is that subsequent germination of stored seed is not impaired by treatment (Gupta et al., 1989).

The traditional use of neem may differ in the region or with farmers of different cultural backgrounds. For example, in southern Sind, Pakistan, farmers mix neem leaves with grains stored in gunny bags, or they rub crushed neem leaves on the inner surfaces of mud bins before filling them with grain. In central Sind, where "palli" made of plant materials is a common storage structure, crushed neem leaves are mixed with mud and used as plaster for its inner sidewalls and top. A survey of various types of on-farm storage practices revealed that a combination of two or three insect control measures, including the use of neem leaves, was used by 29% of the farmers in Punjab and 47% of the farmers in Sind (Borsdorf et al., 1983). In Sri Lanka, farmers burn neem leaves to generate smoke for fumigation against insect pests that attack stored paddy (rice) and pulses (Ranasinghe, 1984). In Nigeria, the traditional use of plant derivatives, including neem, for protecting stored grain has also been documented (Giles, 1964).

The traditional use of neem derivatives is simply based on experience and the understanding that comparatively less damage occurs in the treated stored commodity. However, on scientific grounds, it is clear that in traditional uses, very large quantities of neem material are involved which presumably affect the storage environment. They produce a repellent odour which makes the insect restless and their bitter taste renders treated grain unpalatable to insects. It is desirable to know the amount of various material that provide adequate protection and it is also important to know how neem derivatives affect insect behaviour, growth and development, and reproduction.

Neem seed kernel powder (NSKP) has been tested by Jotwani et al.(1965) against khapra beetle, *Trogoderma granarium*, lesser grain borer, *Rhyzopertha dominica* and rice weevil, *Sitophilus oryzae* . The results showed that NSKP when mixed with wheat at the rate of 1 to 2 parts/100 parts (w/w) of wheat seed afforded very satisfactory protection of wheat seed against all the pests. The protection lasted for at least nine months. No adverse effect on seed germination was observed. NSKP at the above concentration effectively protected mungbean, gram, cowpea, and peas against *Callosobruchus maculatus* for period ranging from eight to eleven months (Jotwani et al., 1967). Germination tests did not show any impairment due to the treatment. The tasting and smelling of the seeds were carried out for the washed and cooked seeds by a panel of six persons. No taste or smell of neem could be detected by any of the tasters. NSKP when admixed with jowar, *Sorhgum vulgare,* seed at 1 to 2.5 parts/100parts of the seed, protected the seed for 6 months against damage by *Sitophilus oryzae* (Singh, 1993).

Application of neem oil at a low concentration of 0.1% to wheat grain greatly reduced egg laying by *Sitotroga cerealella* and was comparable with 5% malathion dust treatment (Verma et al., 1985). Devi et al. (1982) reported that neem oil at 1% afforded protection to stored paddy against *Rhyzopertha dominica* for 6 months. Deoiled neem seed kernel powder, mixed with wheat flour, completely arrested growth

and development of larvae of both *S. aerealella* and *R. dominica* (Singh, 1993). *Trogoderma granarium* was reported to be highly susceptible to the deoiled kernel powder. Concentrations as low as 0.06% part/100 parts of wheat seed prevented development of first instar larvae (Singh et al., 1986).

The insecticidal action of neem extracts was clearly demonstrated by the field application trials done in Mauritius (Jacobson, 1988), where a comparison was made between the neem seed kernel extracts and the most potent synthetic pyrethroid deltamenthrin ("Decis"). The results showed no significant difference between the two treatments in controlling the diamondback moth on Chinese cabbage.

1.5.3. Other organisms

Neem extracts, apart from their action against insects, can also affect other organisms including nematodes, fungi, viruses, and protozoa. Extracts have proved to be quite effective against nematode attack on plants. These materials have been used in small amounts as root-dip treatment, seed dressing , and foliar application. Neem leaf treatment has been used for root-dip treatment for the control of *Meloidogyne incognita* on tomato (Vijayalakshmi et al., 1985) and bare root-dip treatment with neem cake extract and neem oil also prevented *Meloidogyne incognita* and *Meloidogyne javanica* causing root-knot infections in tomato and eggplant (Abid et al., 1991). Azadirachtin and nimbin (a limonoid present in neem extracts) (Fig.1.2) have been used as seed dressing material for tomato, eggplant and okra against *M. incognita* and *Rotylenchoulus reniformis* , and cabbage and cauliflower against *Tylenchorhynchus brassicae* (Siddiqui et al., 1990). Neem seed powder was used for controlling *M. arenaria* and *Pratylenchus penetrans* on tomato (Rossner et al., 1987) and it was also found to inhibit penetration and development of *Anguina tritici* on wheat (Gokte et al., 1988). It is significant, in countries such as India, with both winter and summer

growing seasons and where soil conditions can vary drastically with area, that the efficacy of neem is consistent all-year round, in all soil conditions (Alam, 1991).

There are numerous instances of the effect of azadirachtin and neem on fungal pathogens, including spore germination and mycelial growth of *Helminthosporium nodulosum* and *Pyriculsria grisea* on finger millet. Acetone extracts of neem are more effective than water extracts (Jagannathan et al., 1988). Muthusany et al., (1988) evaluated the efficacy of neem products against rust disease of groundnut. The seed extract and oil were highly effective in inhibiting spore germination by 96.2 and 96.0% respectively, whereas the neem cake extract showed 87.6% inhibition. Certain fruit rots can cause huge economic losses and their control by neem extracts in the laboratory was very promising (Arya, 1988), encouraging the development of field trials in affected areas. Neem, however, is not universally effective against fungi. Khan et al. (1988) could show no effect of dried neem materials on fourteen common pathogenic fungi (dermatophytes, yeast and moulds).

There are several reports on the use of neem and its products against viruses. Verma (1974) reported that nimbidin and nimbin, obtained from neem oil, were active against viruses. The neem oil was highly effective against *Nilaparvata lugens* and suppressed the transmission of Grassy Stunt virus and Ragged Stunt virus diseases of rice (Saxena et al., 1984). Insect survival and disease transmission decreased with an increase in neem oil concentration. Antiviral activity of azadirachtin was not observed, however, against potato leafroll virus PLRV or potato virus Y (PVY) in tobacco seedlings (Nisbet, 1992). As far as antiprotozoan activity is concerned azadirachtin inhibited microfilarial release from *Brugia pahangi* without affecting the motility or viability of the host (Barker et al., 1989).

It has been recorded that a high dose of azadirachtin is required to have an effect on beneficial insects. For example, low doses of azadirachtin (10 and 20ppm) did not harm the hymenopteran parasitoid *Apanteles glomeratus* , although its host, final

instar *Pieris brassicae* larvae, showed reduced feeding followed by a gradual death (Schumutterer, 1992). The effect of azadirachtin and neem seed extract (NSE) on tephritid fruit flies and their parasitoids have shown that azadirachtin is selective against fruit flies and prevents them from either emerging to the adult stage, or else reduces the survival of those adults which do emerge. Adverse effects of azadirachtin against beneficial organisms can not be discounted, however, as shown by suppression of ecdysis and 100% mortality of *Cotesia congregata*, a hymenopteran parasitoid of *Manduca sexta*, following injection of 10μg azadirachtin into newly ecdysed fourth or fifth instar host larvae of the waps. Later injection of azadirachtin did not, however, interfere with ecdysis of second instar parasitoids (Beckage et al., 1988). NSE was not completely harmless to bees, but serious damage to them in the field appears unlikely if spraying is carried out prior to flowering, thus allowing the use of neem on pollen and nectar-producing plants (Schmutterer et al., 1987).

1.6. Range of effects of neem extracts and azadirachtin

The neem tree is emerging as an important source of insecticides. All parts of the tree are biologically active, but the most effective are seed kernel extracts due to their high concentration of azadirachtin. It is with this terpenoid, isolated from the kernels, that the most detailed scientific work has been done. Its effects include antifeedant activity, growth distrupting effects, and effects on reproduction.

1.6.1. Antifeedant activity

Insects evaluated with neem for antifeedant activity show varying degree of sensitivity to various extracts and pure compounds irrespective of the order or the family of the insect. For example, 0.001% concentration of neem seed kernel suspension (NSKS) caused absolute feeding deterrency against the desert locust, *Schistocerca gregaria* , while 0.05% concentration was needed for the same effect on

the migratory locust, *Locusta migratoria*. Both belong to the same family Acrididae. The difference in sensitivity is 50 times (Singh, 1993).

It has been proposed that two types of antifeedant activity evoked by azadirachtin should be distinguished. Primary (gastatory) antifeedant activity, represented by a regulation of food intake resulting from contact between the antifeedant and the sensory organs of the mouthparts, and secondary (non-gastatory) antifeedant activity observed after ingestion, application or injection of the antifeedant (Schumutterer, 1985) (see section 1.6.2).

The mechanism of action of the primary antifeedant response has been investigated in Lepidopteran larvae and in locusts. Perception of the antifeedant at the sensory level may involve different mechanisms. Azadirachtin may stimulate specific deterrent receptors as in larval *Pieris brassicae* (Schoonhoven, 1982) or the molecule may modify the activity of receptors which are specific for other compounds e.g. phagostimulants. The medial sensilla styloconica on the maxillae of the oligophagous larvae of *Spodoptera exempta* and *Mamestra brassicae* have distinct receptors which are responsive to azadirachtin or sucrose, but there is no peripheral interaction between the receptors. In several polyphagous species, e.g. *Spodoptera littoralis* , interaction occurs between these receptors resulting in a modified neural input from the sucrose receptor if the azadirachtin-sensitive receptor is stimulated (Simmonds et al., 1984).

A variety of preparations has been applied to assess the antifeedant activity of azadirachtin ranging from crude to refined neem extracts (water or oil extracted), to neem enriched extracts, to pure azadirachtin. This large variety of formulations has been applied in many different ways against more than 200 species, e.g. to crops; within artificial diets; on simplified feeding discs; to the insects as sprays; topical applications; by injection or by cannulation, thus hindering direct comparisons of the succeptibility of different insect species to the antifeedant effects. Published data on the

antifeedant effects of azadirachtin and "Margosan-O" are presented in Table-1.4 (Mordue et al., 1993) and reveal a vast range in sensitivity between species.

Mane (1968) screened NSKS against four pests, *Euproctis lunata, Spodoptera litura, Utetheisa pulchella* and *Acrida exaltata. E. lunata* was found highly sensitive, perhaps the most sensitive among the Lepidopterous larvae tested so far with neem seed kernel suspension. The relative efficacy of the water, alcohol, chloroform and acetone extracts of neem seed kernel against *E. lunata* for absolute feeding inhibition has been reported as 0.0156% for the alcohol, 0.0312% for water and 0.0625% for the chloroform and acetone extracts (Babu et al., 1969). These extracts were also tested against the larvae of the three other species. All the extracts failed to give absolute antifeedancy even at 2% concentration.

Table 1.4 The antifeedant effects of azadirachtin (AZAD) and "Margosan-O" (M-O) (0.3%AZAD) on a selected range of insect pest species in laboratory and greenhouse trials (Mordue et al., 1993).

species	Treatment	AZAD content (ppm)	Antifeedancy (%)*
Lepidoptera (larvae)			
Spodoptera frugiperda	AZAD(fd)	1.0	92
Heliothis virescens	AZAD(fd)	1.0	94
Spodoptera littoralis	AZAD(fd)	1.0	95
Choristoneura fumiferana	M-O(ad)	0.3	95
Heliothis virescens	AZAD(ad)	0.07	50
Peridroma plorans	AZAD(ad)	0.4	50
Spodoptera littoralis	AZAD(ad)	10.0	100
Earias insulana	AZAD(ad)	50.0	100
Spodoptera litura	AZAD(t)	50.0	37
Spodoptera frugiperda	AZAD(t)	50.0	43

Peridroma saucia	AZAD(t)	2.4	50
Ostrinia nubilalis	AZAD(t)	24.0	50
Achoea janata	AZAD(t)	1.0	54
Achoea janata	AZAD(t)	10.0	75
spodoptera littoralis	AZAD(sp)	0.06	50
Macalla thyrsisalis	M-O(sp)	20.0	41
Spodoptera frugiperda	AZAD(sp)	600.0	100
Pieris brassicae	AZAD(syst)	30.0	56
Coleoptera			
Epilachna varivestis	AZAD(t)	500.0	100
Diabrotica undecimpunctata howardi	AZAD(sp)	100.0	98
Acalymma vittatum	AZAD(sp)	100.0	98
Lepitinotarsa decemlineata	AZAD(sp)	600	0
Hemiptera			
Myzus persicae	AZAD(ad)	100.0	80
Rhopalosiphum padi and Sitobion avenae	AZAD(t)	250.0	50
Asymmetrasca decedens	M-O(sp)	60.0	50
Myzus persicae	AZAD(syst)	300.0	30
Rhopalosiphum padi and Sitobion avenae	AZAD(syst)	500.0	18
Asymmetrasca decedens	M-O(syst)	60.0	100
Rhodnius prolixus	AZAD	25.0	50
Isoptera			
Coptotermes formosans	M-O(fd)	100.0	49
Diptera			
Liriomyza trifolii	M-O(syst)	10.0	31

Orthoptera			
Schistocerca gregaria	AZAD(fd)	0.07	100
Locusta migretoria	AZAD(fd)	50.0	100
Dissosteira carolina	M-O(fd)	150.0	82
Schistocerca gregaria	AZAD(ad)	0.001	100
Melanoplus sanguinipes	AZAD(ad)	1000.0	0
Eyerpocnemis plorans	AZAD(t)	0.01	44
	AZAD(t)	0.1	85
Diapheromera femorata	M-O(t)	150.0	75
	M-O(t)	600.0	91
Gryllus pennsylvanicus	M-O(t)	150.0	100
Scistocerca gregaria	AZAD(sp)	1.0	50

Treatment applied via: fd, filter paper or glass fibre discs; ad, artificial diet; t, topical treatment to leaves, leaf discs; sp, spray application to crop; syst, systemic application
* Antifeedancy given in relation to control feeding

Neem seed kernel extract at 2%, or 10% leaf extract, have been reported as very effective feeding deterrent against *Achaea janata* in the laboratory (Chari et al., 1985). Cabbage leaves sprayed with 0.4% NSKS significantly reduced the damage by *Pieris brassicae* larvae (Sandhu et al., 1975). Cabbage and cauliflower crops sprayed with NSKS and aqueous extract of leaf showed strong antifeedant activity against the aphid, *Brevicorne brassicae* (Singh & Shama, 1986). Neem seed kernel, seed coat and fallen leaves were extracted with water and ethanol (Singh, 1987). The ethanolic extract was re-extracted successively with hexane, chloroform and methanol. These extracts were tested for their antifeedant efficacy against the desert locust, *S..gregaria.* The neem seed kernel was most active, followed by seed coat and leaves. The water extract deterred feeding nearly as effectively as the ethanol extract indicating thereby

that water can be used as an effective solvent for extracting the seed kernel. The seed coat which constitutes about 50% of the seed weight exhibited high antifeedant property. Water and ethanolic extracts of the seed kernel were evaluated against chafer beetle, *Apogonia blanchardi* (Doharey et al., 1989) and found to inhibit the feeding of the beetle at 2.5 and 1% concentrations respectively. Neem oil extractive and water extract of seed kernel were tested against desert locust (Attri, 1975) and the neem oil extractive was found 40 times less active than the water extract of seed kernel.

From the foregoing it is clear that the antifeedant activity of neem to various insect species varies greatly. The desert locust, *S. gregaria* is the most sensitive insect, and gram pod borer, *Heliothis armigera*, the least, to the antifeedant effect of neem. Concentrations ranging from 0.001% to 4% of various neem seed kernel extracts have generally been found to deter the feeding of most of the insects evaluated so far (Singh, 1993).

It is clear that Lepidoptera are extremely sensitive to azadirachtin and show effective antifeedancies from 1-50ppm, depending upon species. Coleoptera, Hemiptera and Homoptera are less sensitive to azadirachtin behaviourally with up to 100% antifeedancy being achieved only at 100-600ppm, whereas Orthoptera show an enomous range in sensitivity from the most sensitive species tested, *S. gregaria* (effective concentration to reduce feeding by 50% , EC_{50} = 0.05ppm) through the moderate sensitivity of *L. migratoria* (EC_{50} = 100ppm) to the extreme insensitivity of *Melanoplus sanguinipes* (EC_{50} > 1000ppm) (Table 1.4).

A simple and common laboratory bioassay to assess the antifeedancy potency of azadirachtin is the leaf-, filter paper- or glass fibre-disc feeding test, in either a "choice" or "no choice" situation (e.g. Alder et al., 1989; Klocke et al., 1991; Arpaia et al., 1993). These bioassays provide essential data for more elaborate trials in both the laboratory and field. For example, conversion of the protective concentration for 95%

of the crop (PC_{95}) (0.1µg/leaf disc) for *S. frugiperda* from leaf disc trials to an equivalent dose for crop spraying, gave good protection of corn plants (Klocke et al., 1991).

The Formosan subterranean termite, *Coptotermes formosanus*, requires relatively high azadirachtin doses to inhibit feeding. The dose of azadirachtin required was more than 100 ppm to reduce feeding significantly on filter paper when compared against the amount of control filter paper consumed (Garcia et al., 1984). On systemically-treated plants, high concentrations of azadirachtin (> 100 ppm) are required to produce primary antifeedant effects on aphids (Griffiths et al., 1978; Nisbet et al., 1993). Azadirachtin concentrations of more than 250 ppm prevented cereal aphids (*Rhorholpalosiphum padi* and *Sitobion avenae*) from setting on systemically-treated barley seedlings although lower doses (50ppm) produced the same effects when applied topically to the leaves. These treatments increased locomotory behaviour and reduced probing frequency of the aphids for up to four days (West et al., 1992).

1.6.2. Secondary antifeedant effects

Some of the toxic effects of azadirachtin are closely related with the feeding. They are the disturbance of hormones, poisoning of the gut, other physiological symptoms related to feeding and difficult to distinguish from other internal effects of the compound.

1.6.2.1. Insect Growth Regulatory (IGR) effect

Growth regulatory effect is the most important physiological effect of neem on insects. It is because of this property that it has emerged as a potent source of insecticides. Rarely have insects not succumbed to this biological effect of neem. The seed kernel of extracts or pure azadirachtin when fed or applied at juvenile stages, arrest

their growth. Depending on dose, the insects are either killed before reaching adult stage, or produce malformed and miniature adults. Other physiological effects recorded are: prolongation of larval period (up to 2 months), production of larval-pupal and pupal-adult intermediates.

McMillan et al. (1969) were the first to report the growth distrupting effect of a chloroform extract of the leaves of *Melia azedarach* , a close relative of neem, against *Spodoptera frugiperda* and *Heliothis zea.* Later Gill et al. (1971) reported that *Pieris brassicae* larvae fed on foliage treated with neem kernel extract failed to develop to maturity and most of them died while moulting. Ruscoe (1972) first described the growth-disrupting effects of azadirachtin in *P. brassicae* larvae feeding on cabbage leaves treated with the compound at concentrations which were not antifeedant. Final instar larvae which had fed on leaves treated with 50ppm azadirachtin were unable to complete their pupal moult. When the larvae had fed on leaves treated with 12.5ppm azadirachtin some were able to complete the pupal moult, but the pupae showed malformations such as larval heads and distended wing pads. These pupae died prior to the imaginal moult. According to the reviews of Jacobson (1986) and Warthen (1989) the growth-disrupting effects of neem seed extracts have been reported for forty-one species of insects covering almost every economically important order. Lepidopteran insects appear to be the most susceptible to the growth-disrupting effects and comprise nearly 40% of the species for which these effects have been described.

Singh (1984) reported growth retarding effect of a water extract of a deoiled kernel powder on larvae of *Culex fafigans.* A concentration of 62.5ppm was needed to arrest the growth of the 2nd instar larvae.

The IGR effects of azadirachtin on different insect species are presented in Table 1.5 (Mordue et al., 1993). The growth-disrupting effects of azadirachtin on larvae of the Mexican bean beetle *Epilachna varivestis* are typical of many other species and are as follows (Rembold, 1988). When larvae feed on treated substrates, they may

remain in the larval state without progressing to the pupal moult. Those larvae which reach the pre-pupal stage may die from desiccating. If metamorphosis to a normal pupa occurs, the resulting adult may be unable to shed the pupal integument. If the adult emerges it usually has deformed wings or mouthparts and dies within a few hours.

Table 1.5 The insect growth regulatory (IGR) effects of azadirachtin on insects after injection, oral cannulation or topical application (Mordue et al., 1993)

Species	Treatment at day0-1 of instar	Azadirachtin concentration µg/insect	µg/g	Moult inhibition and mortality(%)
Lepidoptera				
Heliothis virescens	injection	0.25	(1.7)	15
Spodoptera frugiperda	injection	0.25	(1.5)	45
Spodoptera littoralis	injection	0.25	(1.9)	35
Spodoptera litura	injection		1.1	50
Achoea janata	injection		4.1	50
Peridroma saucia	injection		1-2	50
Bombyx mori	injection		1-2	50
Spodoptera littoralis	oral cann.	0.25	(1.5)	50
Spodoptera frugiperda	oral cann.	0.25	(1.9)	50
Heliothis virescens	oral cann.	1.0	(5)	100
Spodoptera littoralis	topical applic.	0.25	(1.5)	15
Spodoptera frugiperda	topical applic.	0.25	(1.9)	20
Heliothis virescens	topical applic.	0.25	(1.7)	5
Hemiptera				
Rhodnius prolixus	injection	0.0035	(0.2)	50
Oncopeltus fasciatus	injection	0.015	(0.6)	50
Oncopoltus fasciatus	topical applic.	0.0035	(0.14)	50

Orthoptera			
Schistocerca gregaria	injection	1.7	50
Locusta migretoria	injection	1.3	50
Melanophus sanguinpes	injection	3.2	50
Melanoplus sanguinipes	oral cann.	11.3	50
Melanoplus sanguinipes	topical applic.	4.5	50

Over-aged nymphs which have a greatly extended instar may survive for several weeks, e.g. *L. migratoria* (Mordue et al., 1985) and *O. fasciatus* (Dorn et al., 1986) or several months, e.g. *Rhodnius prolixus* (Garcia et al., 1984) beyond the normal instar length of several days, and have been used extensively to investigate the onset of maturation with chronological age. Such insects have not metamorphosed and thus have not achieved the imaginal competence for adult physiology and development (Pener et al., 1987; Pener et al., 1989; Van de Horst et al., 1989). *L. migratoria* over-aged nymphs do, however, achieve partial adult competence despite the lack of moulting and metamorphosis as demonstrated by male mating behaviour (Pener et al., 1987) and the competence to respond to adipokinetic hormone (AKH) (Pener et al., 1989).

Schluter et al., (1985) observed that final instar *Manduca sexta* larvae experienced a dose-dependent reduction in weight gain after injection with azadirachtin. Larvae treated with the highest concentrations of azadirachtin moulted to supernumerary larvae, an effect which can also be produced by starvation. Similarly, Koul et al., (1987) found that final instar *B. mori* larvae injected with azadirachtin experienced a dose-dependant reduction in weight gain resulting in a failure to pupate in those larvae which did not reach a threshold weight of 2.5g.

1.6.3. Effect on reproduction

The interruption of insect reproduction is an important and potent effect. Adverse effects in ovarian development, fecundity and fertility have all been reported (Karnavar, 1987). In the Heteroptera, topical application of methanolic neem seed extracts to final instar *Dysdercus fasciatus* nymphs resulted in adult females which produced only 59% of the eggs produced by untreated females (Jacobson, 1988). Topical application of azadirachtin to *Oncopeltus fasciatus* females produced complete sterility (Dorn et al., 1986). As in larval insects, adults may suffer weight loss or reduced weight gain after azadirachtin treatment. For example, injection of azadirachtin into female *L. migratoria* 2-13 days after the adult moult resulted in a smaller ovaries and a 50% weight reduction compared to untreated females (Rembold, 1988).

Marked reduction in fecundity was observed in *Spodoptera litura* following azadirachtin application. Untreated moths laid as many as 7,923 eggs whereas only 705 eggs were laid by five pairs of azadirachtin treated moths, the reduction being almost 90%. The untreated moths laid eggs up to seven days while the treated females had delayed oviposition and laid eggs only for two days. The egg hatchability in treated moths was only 25% as against 75% in control (Gujar et al., 1984).

Injection of 1µg azadirachtin into red cotton bug, *Dysdercus koenigii* caused 50% mortality by the seventh day of treatment. The surviving insects showed a significant effect on reproduction behaviour and generally no eggs were laid due to failure of vitellogenesis. Topical application of azadirachtin severely impaired embryogenesis (Koul, 1984).

A pronounced reduction of egg output and egg viability occurred in rice moth, *Corcyra cephalonica,* following exposure to neem oil vapour emanating from paper discs containing 160 µl of oil. The total number of eggs laid per treated female was 245.4 as against 401.2 in the control. The mean number of viable eggs laid per treated and untreated female was 231.6 and 360.6 respectively (Pathak et al., 1985).

Therefore, it is clear that neem oil has also insecticidal properties although it does not contain large amount of azadirachtin.

Singh et al (1987) found that *S. gregaria* laid a significantly smaller number of eggs in sand treated with 0.1, 0.5 and 1% concentration of neem seed kernel suspension than the untreated control. Root dipping in 5% neem seed kernel extract reduced the oviposition by *Nephotettix virescens* to 7.6 eggs in the treatment in contrast to 50 in control (Kareem et al., 1988). Ethanol extracts of the petroleum extract of neem seed kernel was reported to deter oviposition of fruit flies, *Dacus dorsalis* and *D. cucurbitae* and the latter was found more sensitive than the former (Singh et al., 1983). It has been reported that an extract from seed kernel caused 90% oviposition deterrency of *Heliothis armigera* on chickpea under laboratory conditions (Singh, 1993).

1.7. Mode of action of azadirachtin

As discussed before, azadirachtin was first purified from crude neem extracts by following its feeding inhibitory activity in *Schistocerca gregaria* (Butterworth et al., 1968). This antifeedant activity has been demonstrated for several other insects species. However, it was found by the quantitative *Epilchna* bio-assay, that this inhibitory effect is dose-dependent, diminishes with reduced concentrations, and is even absent at concentrations which still induce malformations and growth inhibition (Rembold, 1988). Salannin , another compound from neem seeds, is a highly potent feeding deterrent for house flies but it does not interfere with growth and development, whereas azadirachtin does both, depending on the amount of substance taken up (Rembold, 1988). Azadirachtin inhibits feeding at much lower concentrations in hemimetabolous than in holometabolous insects. On the other hand, even in the heteropteran *Rhodnius prolixus*, only high doses of azadirachtin had antifeedant effect

when given through a blood meal, whereas molt inhibition was observed at hundred- to thousandfold lower doses (Garcia et al., 1984).

All the facts show that azadirachtin mainly interferes with the neuroendocrine system, but other physiological effects have also been found.

1.7.1. Endocrine effects

Azadirachtin, apart from its antifeedant effects, was also found to cause major disorders in some phytophagous insects. Treatment of the insects and/ or their food with the pure compound or with azadirachtin-containing extracts caused growth inhibition, malformations, mortality, and reduced fecundity. Similar morphogenetic defects could also be induced by synthetic hormone mimics and it was concluded that azadirachtin might function like the ecdysteroids, which are known to be present in many plants (Rembold, 1988).

A major action of azadirachtin is to modify haemolymph ecdysteroid titres. There is a pronounced effect of azadirachtin on control of ecdysteroid titre as first demonstrated in fifth instar *Locusta migratoria* (Sieber et al., 1987). This effect was introduced as an interference of the compound with the neuroendocrine system of the larvae. This argument is supported by histological studies which clearly show an increase of paraldehyde-fuchsin stainable material in the neurosecretory cells of the pars intercerebralis of azadirachtin-treated last instar locusts (Rembold et al., 1981 and Sieber et al., 1987). Azadirachtin is extremely effective in regulating growth and behavior in *Locusta* at low doses, and feeding inhibition is not the primary cause for growth disruption. Inhibition of ecdysis in azadirachtin-treated larvae is due to interference with the hormonal control of moulting. The ecdysteroid titre in the treated larvae reveals a close relationship between endocrine conditions and morphogenetic processes. A shift, or even complete disappearance of the moulting-hormone titre is the result of azadirachtin application (Sieber et al., 1987). Sieber et al also demonstrated

that, although the overall ecdysteroid titre in azadirachtin-treated larvae was not reduced, the contribution of 20-hydroxyecdysone (20-HE) to the peak was diminished. Macro et al., (1990) have shown that the peak ecdysteroid level in *Tenebrio molitor* pupae injected with azadirachtin was reduced by more than 50%. This reduction was also attributed to diminished 20-HE levels rather than ecdysone levels, which were similar to those found in untreated pupae. In the blue bowfly *Calliphora vicina* , injection of mature larvae with azadirachtin produced a slight delay in the ecdysteroid peak associated with the early pupal stage and the mid-pupal peak was diminished and delayed for several days. Azadirachtin did not inhibit the synthesis of ecdysone but the release of the hormone from the ring gland was prevented. Furthermore, azadirachtin could neutralise the effects of atropine and picrotoxin which stimulate the release of ecdysone from ring glands *in vitro*. Ecdysone 20-mono-oxygenase from the mid gut and fat body is the insect cytochrome P-450 dependent hydroxylase responsible for the conversion of ecdysone to its more active metabolite, 20-hydroxyecdysone. Formation of 20-hydroxyecdysone by 20-mono-oxygenase, is affected within 1 hour of exposure to azadirachtin (Bidmon et al., 1987; Smith et al., 1988). The half life of ecdysone *in vitro* is significantly increased by 10^{-5}M azadirachtin in *C. vicina* in association with a decrease in levels of 20-mono-oxygenase (Bidmon et al., 1987). Cytocrome P-450 levels, are also significantly decreased in mid guts of fifth instar *S. gregaria* and *L. migretoria* orally cannulated with 25µg azadirachtin daily for 4 days (Cottee, 1984). In addition, in *Drosophila melanogaster*, *Ades aegypti* and *M. sexta* 50% inhibition of 20-mono-oxygenase was brought about by 10^{-4} to 4 x 10^{-4}M concentrations of azadirachtin, although, as it was shown, these concentrations are an order of magnitude higher than that needed to achieve moult inhibition and alterations of ecdysteroid titre in the relevent species (Smith et al., 1988).

Juvenile hormone (JH) synthesis is also affected by azadirachtin. Malczewska et al., (1988) demonstrated that treatment of final instar *Galleria mellonella* larvae with

azadirachtin caused a reduction in cold-induced elevation of JH levels, preventing the super-numerary moults observed in untreated larvae.

Inhibition of ecdysis in azadirachtin-treated insects is therefore probably caused by interference with the hormonal control of moulting which is closely linked with weight gain through a secondary antifeedant effect. For example, Kraus et al., (1987) found that *E. varivestis* larvae which had fed on a diet containing azadirachtin, at concentrations which did not produce primary antifeedant effects, rapidly reduced their feeding rate even after transfer to untreated diets, resulting in mortality within one week.

Schluter et al., (1985) observed that final instar *Manduca sexta* larvae experienced a dose-dependent reduction in weight gain after injection with azadirachtin. Larvae treated with the highest concentration of azadirachtin moulted to supernumerary larvae, an effect which can also be produced by starvation. That azadirachtin blocks juvenile hormone release is indicated strongly by the selective destruction of larval crochets or hooked setae of the prolegs in *M. sexta* (Reynold, et al., 1986; Beckage et al., 1988). In *Manduca* crochet epidermis remains competent to moult until the juvenile hormone titre declines on day 2 of the last larval instar (Riddiford, 1981). In response to the decline, the epidermis loses its ability to make cuticle and subsequently dies when next exposed to ecdysteroids, even when juvenile hormone is present again. The critical timing of azadirachtin treatment (prior to the first ecdysteroid peak) and the inability of such tissue to synthesis new crochet cuticle as monitored by subsequent implantation into untreated hosts, strongly suggests a momentary lack of juvenile hormone at this critical period, perhaps from a lack of release of neurosecretory allatotropins (Reynolds et al., 1986).

1.7.2. Other physiological effects

It has been shown that azadirachtin inhibits mitosis in germinal discs and other areas of rapid mitosis, e.g. epidermal cells, midgut epithelial cells, ovary, testis, etc. It was demonstrated using colchicine, that azadirachtin affects mitosis directly in fourth instar larvae of *E. varivestis* (Schluter, 1987). The injection of 0.5μg azadirachtin into 20h L_4 *Epilachna* , a stage at which rapid cell division and growth of wing discs is occurring, results in almost complete degeneration of the wing discs within 24h. Injection of 1μg of colchicine at 19h also reveals degenerated wing discs, with a few cells fixed with chromosomes at metaphase. If the injection of colchicine is given at 4h post azadirachtin treatment, followed by fixation at 24h, very little degeneration of wing discs occurs and many cells are seen with chromosomes in the metaphase arrangement. It is suggestive that cell death in these dividing cells due to azadirachtin treatment is occurring at some stage post metaphase, since cells are reaching that stage as witnessed by the colchocine treatment. Also, in *Tetrahymena thermophila* , a ciliate protozoan, Fritzache et al (1987) showed cell proliferation to be inhibited by azadirachtin, with RNA synthesis being strongly affected.

Azadirachtin has subtle effects which may provide clues as to its cellular mode of action. For example, adult locusts treated with azadirachtin become sluggish and show reduced locomotory and flight activity (Schmutterer, 1990; Wilps et al., 1992). Such a reduced 'tendency' to fly results in a significantly reduced elevation of blood lipids after flight activity compared with controls, which cannot be increased by adipokinetic hormone (AKH) (Wilps et al., 1992). Insect muscle structure has been shown to be affected by azadirachtin. Histological studies of midgut muscle of *S. gregaria* and *L. migratoria* show that the muscle becomes swollen and disrupted in a dose-dependent and time-dependent manner after azadirachtin treatment (Nisiruddin et al., 1993; Cottee, 1984). Ultrastructurally the mitochondria appear swollen and often burst, although the arrangement of the myofibrils does not appear to be disrupted

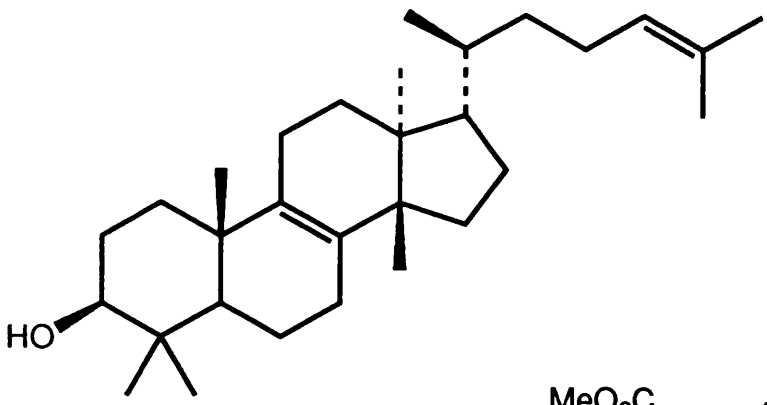
(Nisiruddin et al., 1993). Treated nymphs which die at the moult are unable to swallow enough air to create the correct hydrostatic pressure for ecdysis (Mordue et al., 1986) and the hindguts of such insect *in vitro* are in a more flaccid and extended condition than the controls (Helliwell et al., 1991).

Insects are subject to attack by micro-organisms and parasites and have immune reactions employing a variety of mechanisms, including haemocytes for phagocytosis or encapsulation, antibacterial polypeptides and prophenoloxidases. In *R. prolixus*, apart from the prophenoloxidase systems where melanin production was not reduced, it was found that azadirachtin at a concentration of 1.0μg/ml in the blood meal caused a reduced immune response within 6 days in fifth instars challenged with *Enterobacter cloaca* B12 strain (Azambuja et al., 1991). Protein synthesis in brain, corpus cardiacum, haemolymph and suboesophageal ganglion (SOG) in *S. gregaria* has recently been shown to be affected by azadirchtin treatment (Annadurai et al., 1993). Two-dimensional electophoresis revealed polypeptide profiles showing mainly disappearance but also induction of proteins within 3 days of treatment (2.5μg/g azadirachtin) of adult female *S. gregaria.*

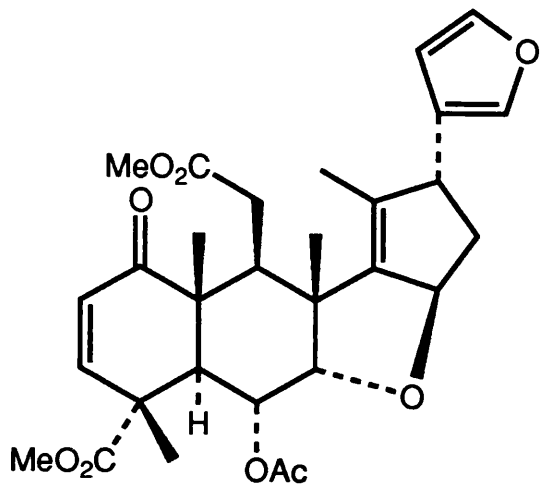
1.8. Chemical properties of azadirachtin

Azadirachtin (fig.1.2), is the most interesting of the tetranortriterpenoids from the neem, and it is found at its highest concentrations in the seeds of the neem tree. Neem oil, which may comprise up to 40% of the seed weight (Jacobson, 1986), contains the less polar triterpenoids e.g. nimbin and salannin (Fig.1.2) and sulphur-containing compounds which give the oil an unpleasant garlic odour.

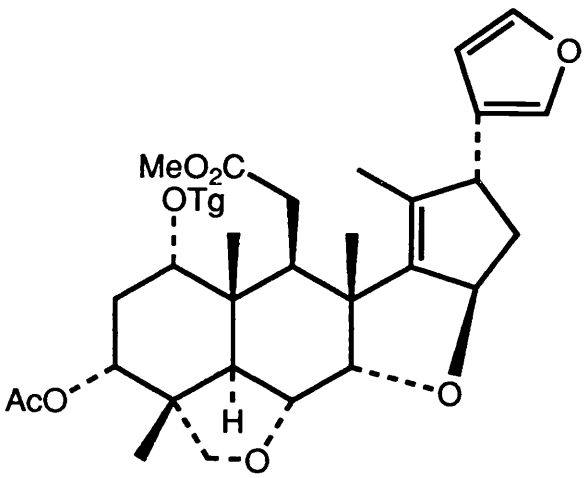
Fig. 1.2. Limonoids from neem seeds and their biogenetic precursor tirucallol



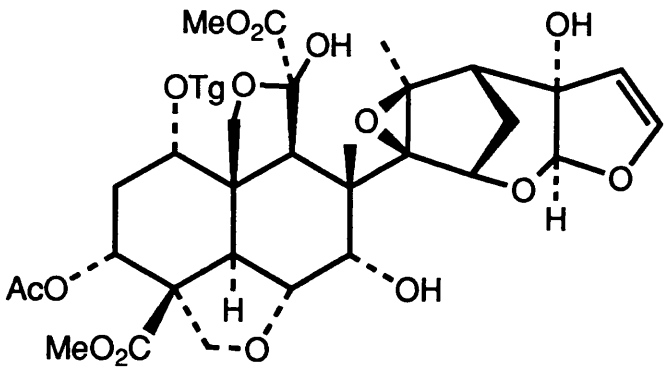
Tirucallol



Nimbin



Salannin



Azadirachtin

Although azadirachtin occurs at concentrations of 0.02%-0.35% (w/w) in the seed (Van Beek et al., 1986), neem oil pressed from the seed usually contains the compound at relatively low and variable concentrations e.g. 0.005%-0.4% (w/v) (Isman et al., 1990). Azadirachtin was discovered in 1967 by Butterworth and Morgan by following antifeedant activity for the desert locust *Schistocerca gregaria* in whole seed extracts. By column and thin layer chromatography, a colourless, microcrystalline solid was isolated with remarkably potent antifeedant activity at a concentration of 70μg/l for the desert locust (Butterworth et al., 1968). Morgan recognized the importance of the discovery and extended the work to identify the functional groups and attempted to obtain a truly crystalline derivative. Although it was discovered in 1967, only 19 years later, in 1986, was the correct structure of azadirachtin reported. Zanno et al. (1975) presented the first incomplete structure proposal. The correct structure appeared in papers submitted in 1985 by Kraus et al. (1985) and Broughton et al.(1986) and the full details of the structure of azadirachtin were finally published in 1987 (Bilton et al., 1987; Kraus et al., 1987; Turner et al., 1987). Azadirachtin is a highly oxidised limonoid with many reactive functional groups in close proximity to each other and a molecular formula $C_{35}H_{44}O_{16}$ (relative molecular mass of 720). It is freely soluble in polar organic solvents and sparingly soluble in water (Butterworth et al., 1971). Its biosynthesis is thought to involve tirucallol (Fig.1.2), a tetracyclic triterpenoid, and a series of oxidation and rearrangement reactions which produce finally, among others, the tetranotriterpenoids salannin, nimbin, and azadirachtin (Ley et al., 1993). Azadirachtin, (also termed azadirachtin A) (Rembold, 1988), is the major terpenoid component of neem seeds. 3-Tigloylazadirachtol (azadirachtin B) is present at concentrations up to 20% of that of azadirachtin. Azadirachtin B (Table 1.6) differs from azadirachtin A and each of the remaining isomers (C-I) (Table 1.6) comprise less than 0.2% of the mixture. Azadirachtin H and I (Table 1.6) have recently been isolated from neem seeds in less yield (4.0% and 1.2% of the total azadirachtin content

respectively) by Govindachari et al., (1992) but the biological activity of these molecules has not been described.

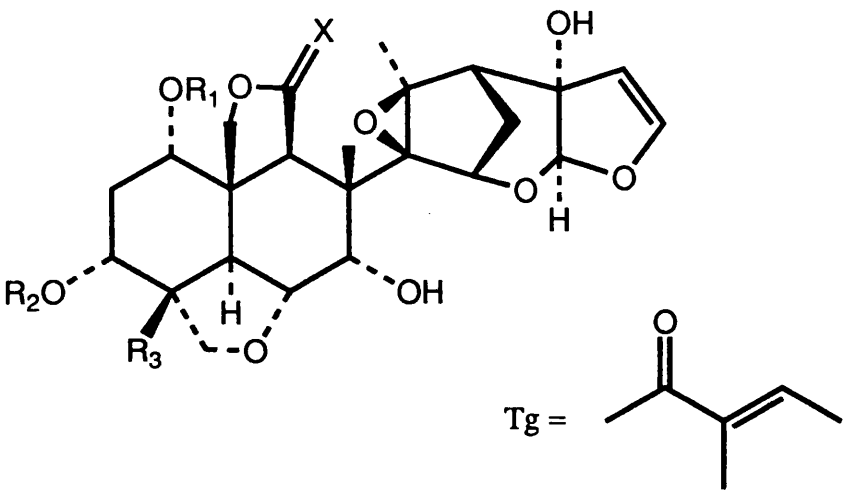


Table 1.6 - Congeners of azadirachtin isolated from neem seed kernel

			Substituents		Molecular	
Name	R_1	R_2	R3	X	formula	weight
Azadirachtin A	Tg	Ac	COOMe	α-COOMe ß-OH	$C_{35}H_{44}O_{16}$	720
Azadirachtin B	H	Tg	COOMe	α-COOMe ß-OH	$C_{33}H_{42}O_{14}$	662
Azadirachtin C	Tg	Ac	COOMe	α-COOMe ß-OH	$C_{33}H_{42}O_{14}$	662
Azadirachtin D	Tg	Ac	Me	α-COOMe ß-OH	$C_{34}H_{44}O_{14}$	676
Azadirachtin E	Tg	Ac	COOMe	α-COOMe ß-OH	$C_{30}H_{38}O_{15}$	638
Azadirachtin F	H	Tg	Me	-	$C_{33}H_{44}O_{14}$	664
Azadirachtin G	H	Tg	COOMe	-	$C_{33}H_{42}O_{14}$	662
Azadirachtin H	Tg	Ac	COOMe	-H, -OH	$C_{33}H_{42}O_{14}$	662
Azadirachtin I	Tg	Ac	Me	-H,-OH	$C_{32}H_{42}O_{12}$	618

1.9. Chemical modification of pure azadirachtin

Hydrogenation of azadirachtin facilitates tritium labelling of the molecule. [22,23]-Dihydroazadirachtin has the same biological activity as azadirachtin A (Rembold et al., 1988). Hydrogenation of the 22,23 double bond of azadirachtin with palladium catalyst readily gives dihydroazadirachtin which opens the possibility of studies with [22,23-$^{3}H_2$]-dihydro derivatives (Rembold et al., 1988). [22,23]-Dihydroazadirachtin is a more stable compound with greater potential for field use (Broughton et al., 1986; Yamasaki et al., 1987).

1.10. Aims of the project

As has been shown in this Introduction, a great deal of work throughout the world over the last 30 years has indicated clearly that extracts of the neem tree have a great potential as biological insecticides, following the model of pyrethrin almost a hundred year ago. Although the broad picture is reasonably clear, there is still a great deal of confusion about the detail. There are many reasons for this. One is that much of the work has been done with crude, or partially purified, extracts. It is clear from the reported experiments that there are many active compounds in the various parts of the plant. Very few of these have been purified and characterised. Scientific exactitude requires that experiments be done with pure compounds. Another source of uncertainty is that although neem compounds produce the same effects in many species of insects, there are wide ranges of sensitivity.

Probably the greatest source of uncertainty about how the terpenoids produce their effects on insects is the multiplicity of effects they produce even in one species. In this they contrast with pyrethrin and the synthetic pesticides whose main, and often only, effect, is against the nerve cells. It is clear that azadirachtin and other neem-derived terpenoids can act against many systems and tissues in insects. Many of the

effects produced are associated with effects on hormonal metabolism and release. The complex interactions between the various systems, many of which are poorly understood, make it impossible to draw clear conclusions working only with intact organisms.

The work reported here aimed to address some of these difficulties in the hope of identifying more clearly the mode of action of the terpenoids in specific tissues. The insect *S. gregaria* was employed partly because it has been shown to be particularly sensitive to all the reported effects of neem extracts, but also because its large size enables the isolation of specific tissues whose metabolism can be examined *in vitro*. As discussed above, it was considered important that only purified materials of known provenance should be employed, and so the main compound, azadirachtin, was purified from Sri Lankan neem seed, and fully characterised. The dihyro-derivative, which may found to be a better insecticide due to its greater stability, apparently has the same biological effects as azadirachtin. Substitution of tritium for hydrogen has allowed the development of a suitable tracer for azadirachtin.

A major aim of this project work was to obtain tritiated azadirachtin and use it to answer such questions as

a) ease of cuticle penetration,

b) rate and extent of uptake into specific tissues,

c) extent and location of metabolism,

d) nature of metabolites,

e) rate and extent of excretion.

Although some of this work has been done before, the results have been contradictory, and incomplete.

The results of uptake into specific tissues was the basis for the second part of the work which set out to answer the question of the physiological and biochemical effects of azadirachtin and dihydroazadirachtin on identified tissues. The intention was

to look for a clear direct effect on isolated tissue, which could be used to investigate the intracellular mode of action of the compounds.

CHAPTER 2

EXTRACTION AND PURIFICATION OF AZADIRACHTIN AND SEVERAL OTHER COMPOUNDS FROM SEEDS OF NEEM *AZADIRACHTA INDICA*.

2.1. Introduction

Although all parts of the neem tree have long been known to be resistant to insect attack, the seeds contain most of the active materials which are chemically diverse and structurally complex. Azadirachtin (Fig.1.2), which was first isolated by Butterworth and Morgan (1968), has been shown to be the most active of a whole group of similar insecticides (azadirachtin B-I) (Table 1.6) from neem seeds (Butterworth et al., 1968; Forster, 1983; Rembold et al., 1984; Govindachari et al., 1992).

Several procedures have been published for the isolation of azadirachtin (Butterworth, et al. 1968; Nakanishi, 1975; and Yamasaki, et al., 1986). Purification has been accomplished by successive partitioning between solvents, column chromatography, flash chromatography, preparative thin layer chromatography (TLC), and preparative high performance liquid chromatography (HPLC). It has been found to be advantageous to defat the ground seeds exhaustively with petroleum ether or hexane prior to extraction with methanol. The methanolic extract is then subjected twice to efficient partitioning between methanol and petroleum ether to remove any remaining oils and other non polar materials, followed by partitioning between water and ethyl acetate to remove what appear to be water soluble proteins and sugars. The final step of the purification is flash chromatography, which is an efficient technique for purifying azadirachtin. The method of Still et al. (1978) was modified to use a vacuum-driven system with "wide" (7 cm x 15 cm) or "narrow" (4 cm x 15 cm) columns according to the amount of material to purified. The dry column technique described by Harwood (1985) can also be employed for the purification using a P40 (5 cm length) flash column.

The advantages of flash chromatography are rapid and reproducible separation and simplicity of loading of material and eluting solvents. The flow rate

does not noticeably affect the separation. Solvent can be pulled through at an appreciable rate or left to gravity [ca 20 ml / min] (Schroeder, et al., 1987).

Crystallisation of azadirachtin is carried out using carbon tetrachloride as a solvent. A white microcrystaline material is collected by suction filtration. Characterisation of the compound can be carried out using reverse phased HPLC, H^1-NMR, and C^{13}-NMR.

2.2 Method of extraction

All organic solvents used for the extraction were "Bulk" grade. The solvents used for the flash chromatography and thin layer chromatography were "Analar" grade. Partially purified fractions were stored in sealed containers at -20°C after the complete removal of solvents by evaporation and evacuation on an oil pump.

2.2.1. Extraction of fatty materials from neem seeds.

Neem seeds from Sri Lanka were used for the extraction. Neem seeds (1.150kg) were finely ground and immediately transferred to a large conical flask. The fat extraction of ground seed was then implemented using petroleum ether 60°-80°c (approximately 2.5 l). The mixture was stirred occasionally at room temperature for 3 hours to extract the fatty materials. The petroleum extract was decanted and the process was repeated with fresh n-hexane overnight three more times until no further yellow coloured oil was extracted by the solvent. The pooled hexane extracts were discarded and the remaining defatted seed (marc) was then extracted twice with 1/1 w/v of methanol for 3 hours. This process was difficult, probably because exhaustive oil extraction had left the marc finely fragmented and compacted. The extraction with methanol was repeated for a third time. In this case the mixture was shaken by hand for 5 min at 15 min intervals during a one hour

period. The marc was then filtered using a Buchner apparatus and the methanol filtrate was concentrated by rotary evaporator at 40°C. This procedure was repeated three more times and then overnight extraction with MeOH was carried out again. The pooled methanol extracts were combined and concentrated to give a brown tar.

The brown tar was redissolved in methanol and partitioned three times with equal volumes of hexane to remove any remaining non-polar components. The methanol layer was then taken to dryness on a rotary evaporator and, after being dried under high vacuum, the extract was weighed. The methanol extract yielded 295.5 g of a dark viscous extract.

2.2.2. Partition with ethyl acetate and water.

The dried methanol extract was redissolved in one litre of ethyl acetate, transferred to a 4 l separating funnel, and partitioned with one litre of water. This procedure was repeated and the aqueous layer was again washed with ethyl acetate and all the ethyl acetate layers were combined. The aqueous layer contained all water soluble protein and sugars. The ethyl acetate layer was dried with anhydrous Na_2SO_4 and taken to dryness on a rotary evaporator at 40°C, yielding 24.2 g.

2.2.3. Flash column chromatography

The dried extract was dissolved in 50 ml of dichloromethane and 30 grams of silica was added to the round bottom flask. The mixture was taken to dryness on a rotary evaporator to adsorb the extracted material thoroughly on to the silica. A column (8 cm x 17 cm) was packed with silica (230-400 mesh) and the extract adsorbed on silica, was then added on to the top of the column. The column was first eluted with 40/60 (v/v) ethyl acetate/petroleum ether and 10 fractions of 25 ml were collected. The polarity of solvent was increased gradually as described in Table 2.1.

Table 2.1 The solvent system used in the first flash chromatographic purification of azadirachtin.

Fraction	Solvent system			Number of fractions
	Petroleum ether :	Ethyl acetate :	Methanol	
A	60	40	0	5
B	50	50	0	5
C	40	60	0	5
	30	70	0	5
D	20	80	0	5
	10	90	0	5
	0	100	0	5
E	0	0	100	5

The average fraction size was approximately 25 ml. Forty fractions were collected and were individually chromatographed with pure azadirachtin as standard on a silica TLC plate (10 x 20). The solvent system used for the TLC analysis was 70:30 (v/v) ethyl acetate : petroleum ether. After development, 25% H_2SO_4 acid was sprayed on to the plate which was heated in an oven at 200°C for 5 min. Fractions were combined according to the TLC profiles. Five combined samples (A-E) were collected. The weight of the fractions are shown in Table 2.2. The fraction C was found to contain azadirachtin. They were all taken to dryness on a rotary evaporator.

Table 2.2 Yields of the fractions obtained from the methanol extraction of the neem seeds.

Fraction	Weight (g)
A	20.2
B	5.0
C	8.5
D	3.6
E	12.5

2.2.4. Isolation of azadirachtin from fraction C

Fraction C was redissolved in 5 ml of dichloromethane and adsorbed on 3.3 g of silica as before. It was then applied to a small silica column (2.5 cm x 10 cm). More precise polarity of the eluting solvents was used and smaller fractions were taken, as shown in Table 2.3, to aid purification.

The average fraction size was approximately 10-15 ml. The azadirachtin-containing fractions were pooled according to their purity. Fraction C_1 contained less-polar impurities fraction, C_2 contained mostly azadirachtin and traces of one less polar impurity and fraction C_3 contained traces of a more polar impurity. Those three fractions were purified by preparative TLC using the same solvent system which had been used for the analytical TLC.

Purified azadirachtin was obtained from the preparative TLC of C_2. The material was dissolved in carbon tetrachloride by heating on a steam bath, and the solution was filtered and stored in the freezer. The solution clouded soon after the filtering and did not clear for nearly 12 hours. A white microcrystalline solid was collected by filtration, yielding 754 mg of azadirachtin (1) (Fig. 1.2).

Table 2.3 The solvent system used in the second flash column chromatographic purification of azadirachtin in fraction C.

Solvent system		Number of fractions
Petroleum ether (60-80) :	Ethyl acetate	
60	40	5
50	50	5
40	60	5
30	70	5
20	80	5
10	90	5
0	100	5

2.2.5. Analysis of fraction B

This fraction contained less polar compounds than azadirachtin. It was subjected to flash column chromatography using a narrow column and the solvent system shown in Table 2.4. Average fraction size was approximately 25ml.

Analytical TLC was used to check the purity and fractions were pooled according to their purity (B_1, B_2, B_3). Fraction B_1 contained one compound, fraction B_2 contained two compounds and fraction B_3 contained one compound. The compounds contained in fraction B_2 consisted of a mixture of those present in B_1 and B_3. They were separated by preparative TLC and two pure compounds (2) 200 mg and (3) 80 mg respectively were isolated. Recrystallisation of these products was carried out using ethyl acetate as solvent. Purity was confirmed using

^{1}H and ^{13}C NMR analysis which identified compound (2) as salannin and compound (3) as deacetyl salannin (Fig. 3.6) (Kubo et al., 1986).

Table 2.4 The solvent system used in the third flash column for chromatographic purification of the less polar compounds in fraction B .

Solvent system		Number of fractions
Petroleum ether (60-80)	Ethyl acetate	
60	40	3
50	50	5
40	60	10
30	70	10
0	100	3

2.2.6. Analysis of fraction D

This fraction contained more polar compounds than azadirachtin and was subjected to flash chromatography, using a narrow column and eluting with the solvent shown in Table 2.5.

The average fraction size was approximately 20ml. Fractions were pooled according to their relative purity. After the initial attempted separation, all fractions contained the same polar compounds and were therefore pooled for preparative TLC separation using 95:05 chloroform : methanol as solvent system. One pure compound, white, needle-shaped crystalline material (33 mg) was isolated as a result of the preparative TLC. Identification of the compound was carried out using

^{1}H-NMR and ^{13}C-NMR. Structure (4) was assigned to this new compound (see results). Subsequently a compound with structure (4) was identified as azadirachtin H (Table 1.6.) (Govindachari, et al., 1992).

Table 2.5 The solvent system used in the flash column chromatographic purification of the more polar compounds in fraction D.

Solvent system		Number of fractions
Petroleum ether 60-80	Ethyl acetate	
40	60	5
30	70	15
20	80	15
10	90	5
0	100	2

2.2.6. Confirmation of purity and identification of azadirachtin and congeners.

The purity of each compound was established using the following methods.

(i) Analytical TLC

(ii) Analytical HPLC

(iii) 200 MHz ^{1}H NMR

(iv) Melting point analysis

2.2.6.1. Analytical TLC

Standard azadirachtin (1*) (which was kindly provided by Dr. A.J. Nisbet, University of Aberdeen), purified azadirachtin (2*), and the initial methanol extract which contained other isolated compounds (3*) were chromatographed on a TLC plate. TLC was performed on 10 x 20 plastic silica plates with a 250 μm thick layer of silica containing binder. The solvent system used was 70:30 (v/v) ethyl acetate : petroleum ether. Vanillin reagent (3 g of vanillin, 1 ml of c.H_2SO_4 in 100 ml of absolute ethanol) was used to visualize the spots.

2.2.6.2. Analytical HPLC

A model 802 Gilson liquid chromatography instrument was used for the analysis. The analysis was performed using a 25 cm x 4.6 cm C6 reverse phase column (Spherisorb ODS2, 5 μm partical size) on the following system.

Pump : Gilson 303

Detector : Gilson " Holochrome" UV-VIS detector set at 229 nm.

Detector sensitivity : 0.1 range

Solvent system: 1:1 methanol: water (v/v)

Settings : 1ml/min flow rate, 30 min run time,

100 μl loop.

2.2.6.3. ^1H NMR and ^{13}C NMR

^1H and ^{13}C NMR spectroscopy were performed on a Bruker WP 200 SY instrument. Shifts in ppm are relative to $CHCl_3$ at 7.25 ppm and $CDCl_3$ at 77.0 ppm.

2.2.6.4. Melting point analysis

Melting points of azadirachtin and the other compounds were determined on a Kofler hot stage apparatus and are uncorrected.

2.2.7. Hydrogenation of azadirachtin

A sample (25 mg) of azadirachtin in ethyl acetate (25 ml) was shaken with 10% paladium on charcoal (25 mg) as a catalyst under a hydrogen atmosphere for 30 minutes (This is a modification of the method used by Rembold et al., 1988). The reaction mixture was filtered through celite and the celite was rinsed five times with 5 ml portions of ethyl acetate. The combined filtrates were evaporated to dryness on a rotary evaporator at 40°C. The residue was then recrystallised from carbon tetrachloride.

The purity and identity of the compound were respectively assessed by thin layer and high performance liquid chromatography, and NMR analysis.

2.2.8. Commercial tritiation of azadirachtin.

A sample of pure azadirachtin (50 mg) was sent to Amersham International Ltd., Alyesbury, along with the catalyst and exact instructions as to the method to be used for tritiation. The product was returned dissolved in a total 40ml of ethyl acetate.

2.2.9. Characterisation of the [22,23-$^3H_2$]-dihydroazdirachtin

Dilutions in ethyl acetate (ten, hundred and thousand fold) were made of the material as supplied, and samples subjected to chromatography in order to determine the best working concentration. The purity was determined by chromatography as follows:

2.2.9.1. TLC.

Samples (2 µl) of the tritiated material were co-chromatographed with the unlabelled dihydro compound on silica using ethyl acetate either alone or mixed

with various proportions of petroleum ether (40-60) as solvent. The silica contained a fluorescent agent to facilitate the discovery of UV- absorbent compounds. Subsequently, two dimensional chromatography on the same medium was used, with ethyl acetate/petroleum ether 80/20 (v/v) as solvent in the first direction and ethyl acetate only in the second.

The presence of carrier compound was determined by its absorbance of light from a UV examination lamp, and the presence of radio-activity established by scanning in a TLC scanner. An alternative method of locating the presence of the radio-isotope was to use one-dimensional chromatography as described above, and then scrape 1 cm segments of the silica medium into separate 5 ml plastic scintillation vials and estimate the activity by scintillation counting as described below.

2.2.9.2. HPLC

Reverse phase HPLC chromatography was carried out on a 25 cm C6 "Spherisorb" column using various mixtures of methanol/water as eluent. The presence of the dihydro derivative was detected by its absorbance at 219 nm. Samples (1 ml) of the eluant were collected and the radio-activity estimated by means of scintillation counting. 50 µl aliquots were taken for the scintillation counting.

Quantification of the compound by its absorbance at 219 nm was done by comparison with known amounts of 22,23 dihydroazadirachtin, run under the same conditions.

2.2.9.3. Scintillation counting.

The tritium was estimated by scintillation counting using a counter programmed for quench correction. Radiolabelled material, either in the form of a

solution or absorbed on the silica of the TLC, was added to 3 ml of "Ecoscint", and counted for 1 min. The efficiency of counting was found to be 60%.

2.3. RESULTS

2.3.1. Purification of azadirachtin and other compounds.

Azadirachtin (**1**) (Fig. 1.2) and three other compounds were extracted and purified from Sri Lankan neem seeds. In total 0.754g of azadirachtin was isolated from 1.150kg of the neem seeds. As stated above, the purity was confirmed by analytical TLC, HPLC, melting point analysis and ^{1}H and ^{13}C NMR spectroscopy. The results of the TLC chromatogram of standard azadirachtin (1^{*}), purified azadirachtin (2^{*}), and the methanol extract of neem seeds (3^{*}) are shown in Fig. 2.1.a. The HPLC chromatogram of azadirachtin and the ^{1}H and ^{13}C NMR spectra of azadirachtin are shown in Figs. 2.2, 2.3, and 2.4 respectively. The NMR data and assignments of azadirachtin and dihydroazadirachtin are given in Table 2.6. The melting point of azadirachtin was 150-152°C.

Table - 2.6. ^{1}H-NMR and ^{13}C-NMR shifts and assignments of azadirachtin and dihydro azadirachtin

	Azadirachin				Dihydroazadirachtin
^{1}H atom	Chemical shift & Multipicity	^{13}C atom	Chemical shift & Multipicity	^{1}H atom	Chemical shift & Multipicity
1	4.7(t)	C-1	70.5(d)	1	4.7(t)
2a	2.3(dt)	C-2	29.4(t)	2a	2.28(dt)
2b	2.3(dt)	C-3	66.9(d)	2b	2.19(dt)
3	5.5(t)	C-4	45.4(s)	3	5.43(t)
5	3.3(d)	C-5	37.1(d)	5	3.24(d)

6	4.6(dd)	C-6	74.4(d)	6	4.45(dd)
7	4.7(d)	C-7	76.4(d)	7	4.68(d)
9	3.3(s)	C-8	50.1	9	3.27(s)
15	4.7(d)	C-10	52.5(s)	15	4.63(d)
16a	1.7(ddd)	C-11	104.1(s)	16a	1.67(ddd)
16b	1.3(d)	C-13	69.9(s)	16b	1.49(d)
17	2.4(d)	C-14	68.5(s)	17	2.43(d)
18	2.1(s)	C-15	73.7(d)	18	1.97(s)
19a	4.2(d)	C-16	25.1(t)	19a	4.11(d)
19b	3.6(d)	C-17	48.6(d)	19b	3.58(d)
21	5.6(s)	C-18	20.8(q)	21	5.24(s)
22	5.1(d)	C-19	18.4(q)	22	2.12(m)
23	6.5(d)	C-20	83.5(s)	23a	3.98(td)
				23b	3.87(td)
28a	4.1(d)	C-21	107.3(d)	28a	4.03(d)
28b	3.8(d)	C-22	108.7(d)	28b	3.77(d)
30	1.8(s)	C-23	147.0(d)	30	1.71(s)
OMe	3.8(s)	C-30	72.9(t)	OMe	3.75(s)
OMe	3.7(s)	C-31	173.2(s)	OMe	3.64(s)
OAc	2.0(s)	C-1^1	166.1(s)	OAc	1.91(s)
3'	6.9(q)	C-2^1	128.6(s)	3'	6.85(q)
4'	1.8(q)	C-3^1	137.5(d)	4'	1.74(d)
5'	1.8(s)	C-4^1	14.3(q)	5'	1.81(s)
CH_3	2.7(s)	C-5^1	11.9(q)	OH	2.80
OH	2.8	OMe	52.5(q)		3.19
			53.5(q)		5.03
		OAc	21.3(q)		
			169.5(s)		

The compounds from fractions B_1 and B_2 were identified as salannin (**2**) and deacetylsalannin (**3**) (Fig.3.6) by comparision of their spectroscopic profile data with those of authentic specimens. The melting points of these two compounds were 167-169°C and 206-208°C respectively. The ^{1}H NMR of deacetylsalannin and salannin are shown in the appendix (Kubo et al., 1986).

Fig. 2.1. (a) The figure represents a silica gel TLC of standard azadirachtin (1^*), purified azadirachtin (2^*) and the crude methanolic extract of neem seed (3^*).

(b) The figure represents a silica gel TLC of dihydroazadirachtin (1^*) and standard azadirachtin (2^*).

The chromatograms were run in 70% ethyl acetate: petroleum ether and then, chromatograms were stained with vanillin reagent.

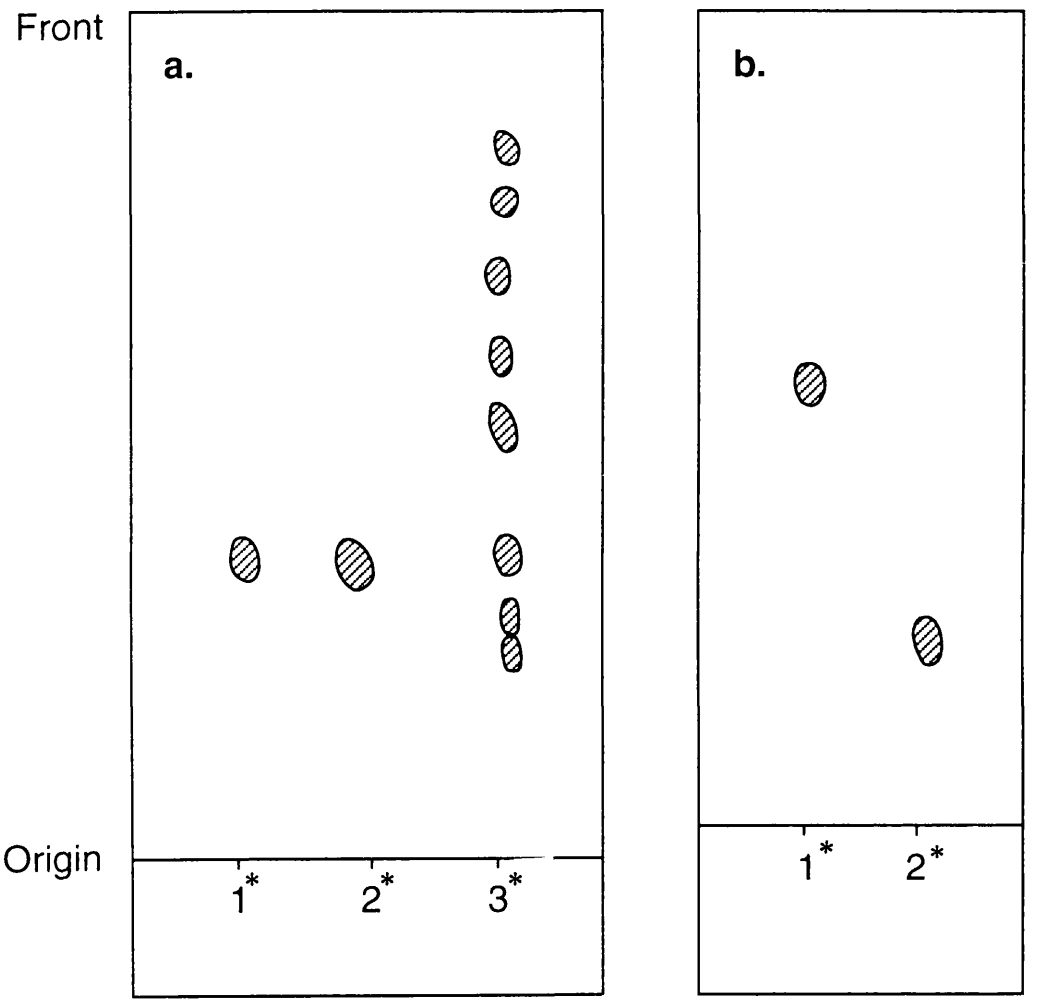

Front
a.
b.
Origin
1*
2*
3*
1*
2*

Fig. 2.2 Analytical high-performance liquid chromatogram of azadirachtin.

The figure represents the chromatography of azadirachtin by reverse phase HPLC under the conditions previously described. The solvent was 50:50 methanol water (v/v). The arrow indicates the point of sample injection.

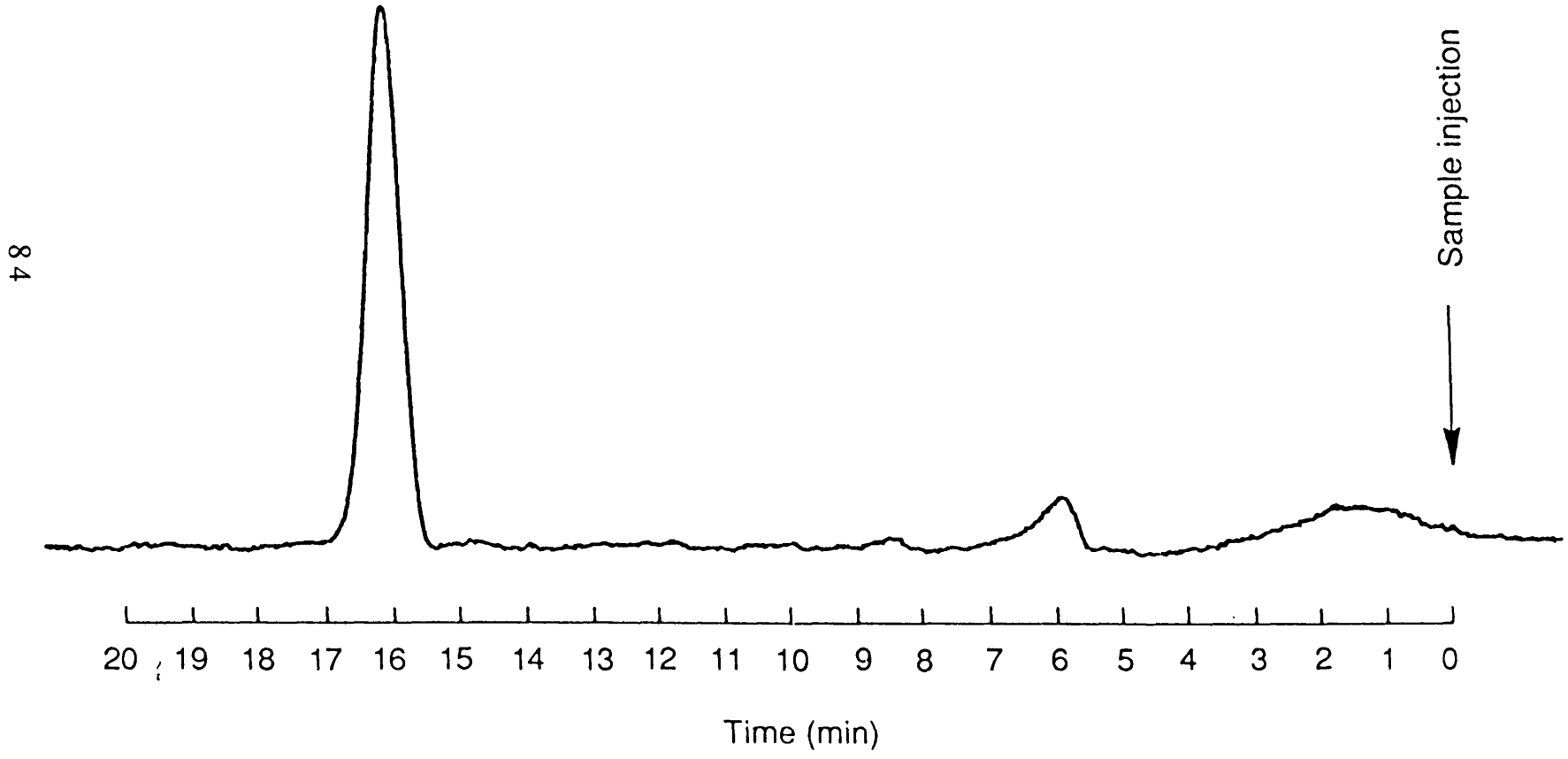

84
Sample injection
20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
Time (min)

Fig. 2.3 ^{1}H-NMR spectrum of azadirachtin.

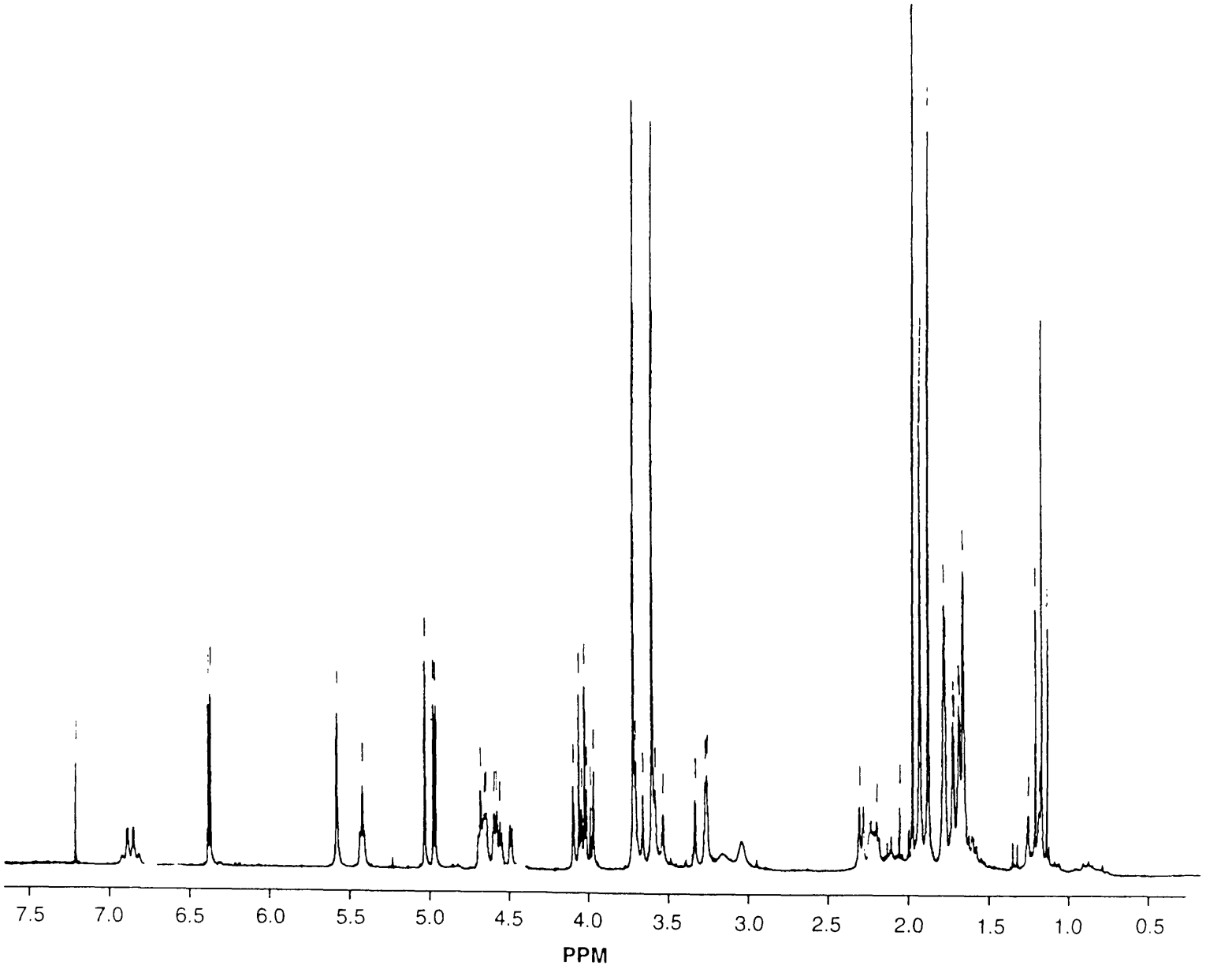
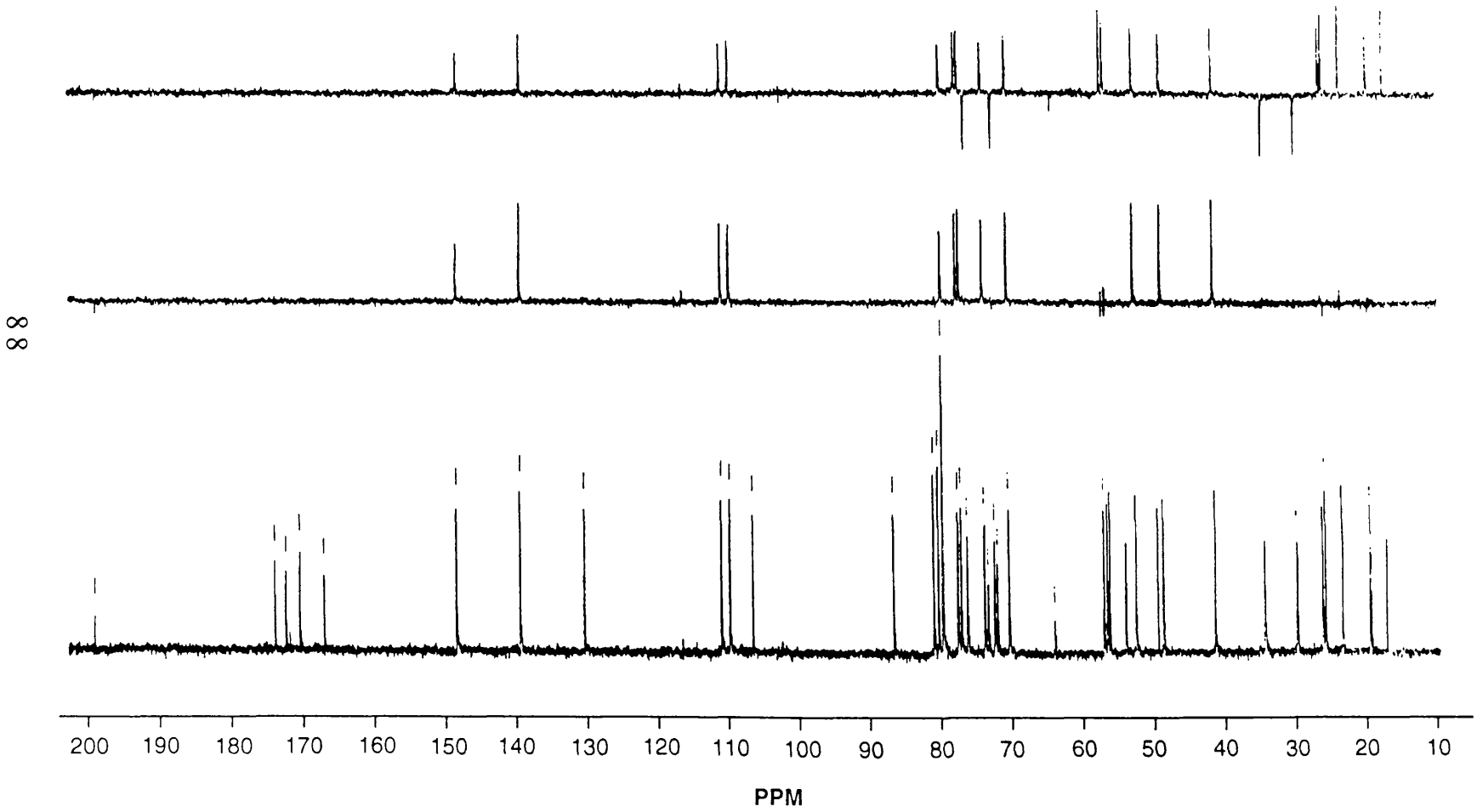

7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
PPM

Fig. 2.4 ^{13}C-NMR spectrum of azadirachtin.


88
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
PPM

The compound from fraction D has melting point 246-248°C and was identified as azadirachtin H (**4**) (Table 1.6). The NMR data and assignments of compound (**4**) are given in the appendix. The yield of dihydroazadirachtin in the hydrogenation experiment was approximately 70%. The purity and identity of the compound were respectively assessed by TLC (Fig. 2.1.b.), HPLC (Fig. 2.5), and ^{1}H NMR analysis (Fig. 2.6). A HPLC chromatogram of both azadirachtin and dihydroazadirachtin is shown in Fig. 2.7.

The unlabelled dihydroazadirachtin was used as a marker for the identification of tritiated dihydroazadirachtin. The purity of the tritiated dihydroazadirachtin (**6**) (Fig. 3.6) was first determined by TLC. The results of a TLC co-chromatogram of unlabelled dihydroazadirachtin and tritiated dihydroazadirachtin, a two dimensional chromatogram on the same medium and a one dimensional chromatogram of tritiated dihydroazadirachtin are shown in Figs. 2.8, 2.9, and 2.10. Reverse phase HPLC was carried out to confirm the purity. Fig. 2.11 shows the HPLC profile of azadirachtin, dihydroazadirachtin, and tritiated dihydroazadirachtin.

2.4. DISCUSSION

In a study of the efficiency and selectivity of different solvents in the extraction of azadirachtin from neem kernels, it was shown that methanol, water, methyl ethyl ketone and the azeotropic mixture of methyl t-butyl ether and methanol were the most efficient (Feurhake, K. J., 1984). The azeotropic mixture showed the best selectivity while the highest degree of purification and enrichment of azadirachtin and other highly active substances was achieved by a liquid-liquid partition of the aqueous extract with methyl t-butyl ether.

Fig. 2.5 Analytical high-performance liquid chromatogram of dihydroazadirachtin.

The figure represents the chromatography of dihydroazadirachtin by reverse phase HPLC under the conditions previously described. The solvent was 50:50 methanol water (v/v). The arrow indicates the point of sample injection.

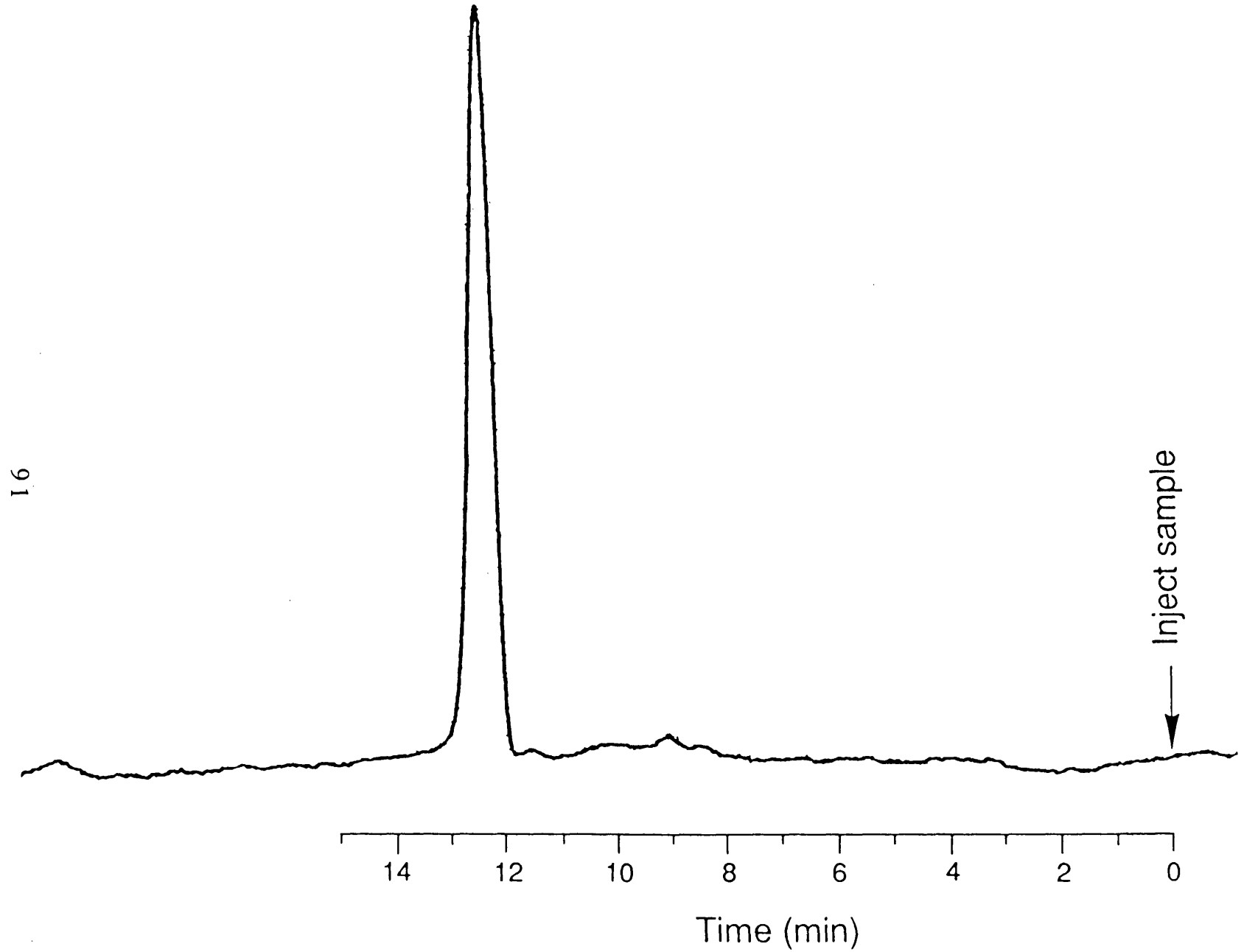

91
Inject sample
14
12
10
8
6
4
2
0
Time (min)

Fig. 2.6 ^{1}H-NMR spectrum of dihydroazadirachtin.

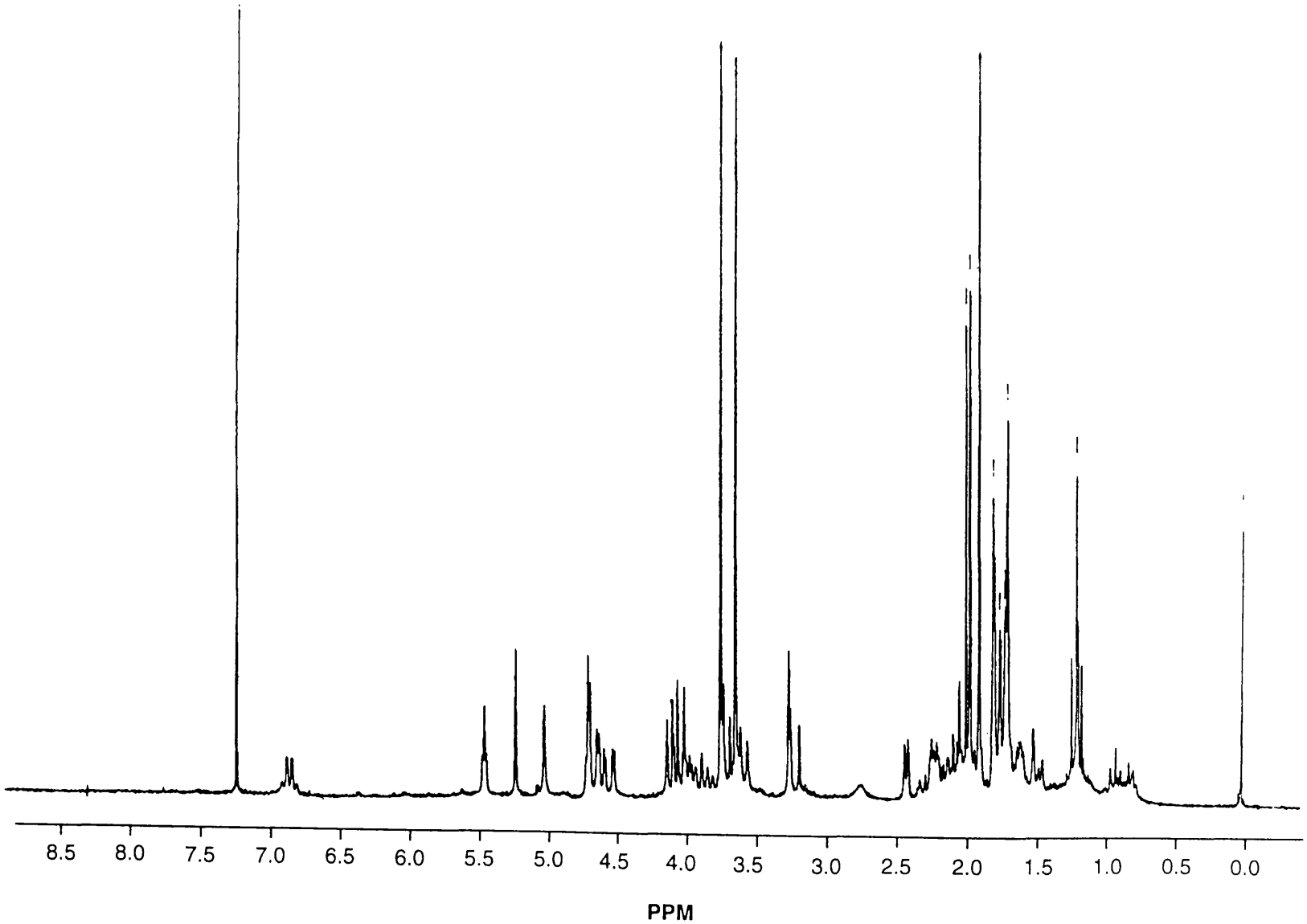

8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PPM

Fig. 2.7 Analytical high-performance liquid chromatogram of azadirachtin and dihydroazadirachtin.

The figure represents the chromatography of azadirachtin (b) and dihydroazadirachtin (a) by reverse phase HPLC under the conditions previously described. The solvent was 50:50 methanol water (v/v). The arrow indicates the point of sample injection.

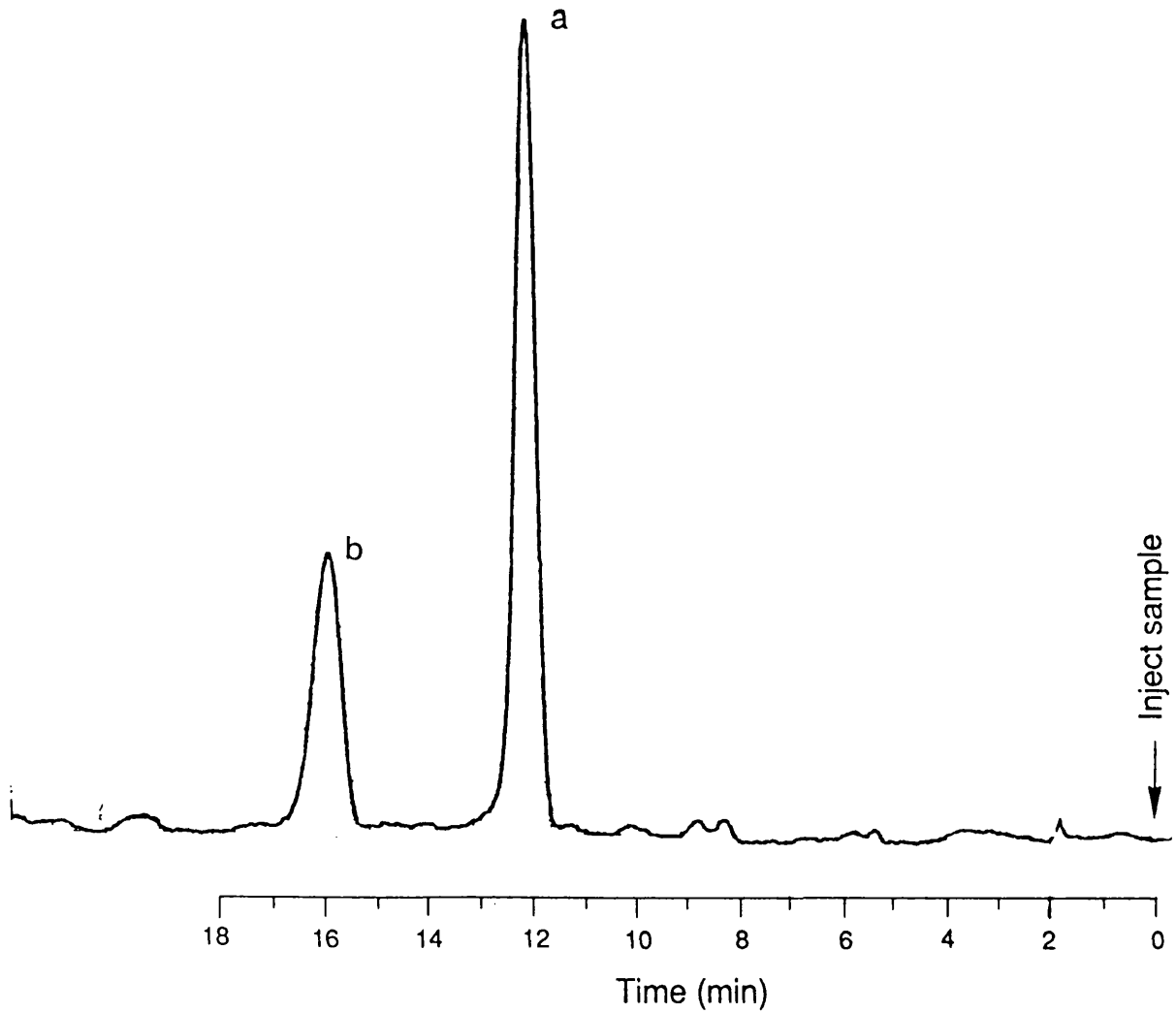

a
b
Inject sample
18
16
14
12
10
8
6
4
2
0
Time (min)

Fig. 2.8. Chromatogram of [22,23-^{3}H] dihydroazadirachtin

The figure represents, to the actual scale, a one-dimensional TLC on silica gel of the radiolabelled product, co-chromatographed with unlabelled [22,23] dihydroazadirachtin. The lower trace indicates the location of the carrier compound, while the upper part shows a scan for the presence of radio-activity. The arrow indicates the point of application of the compounds.

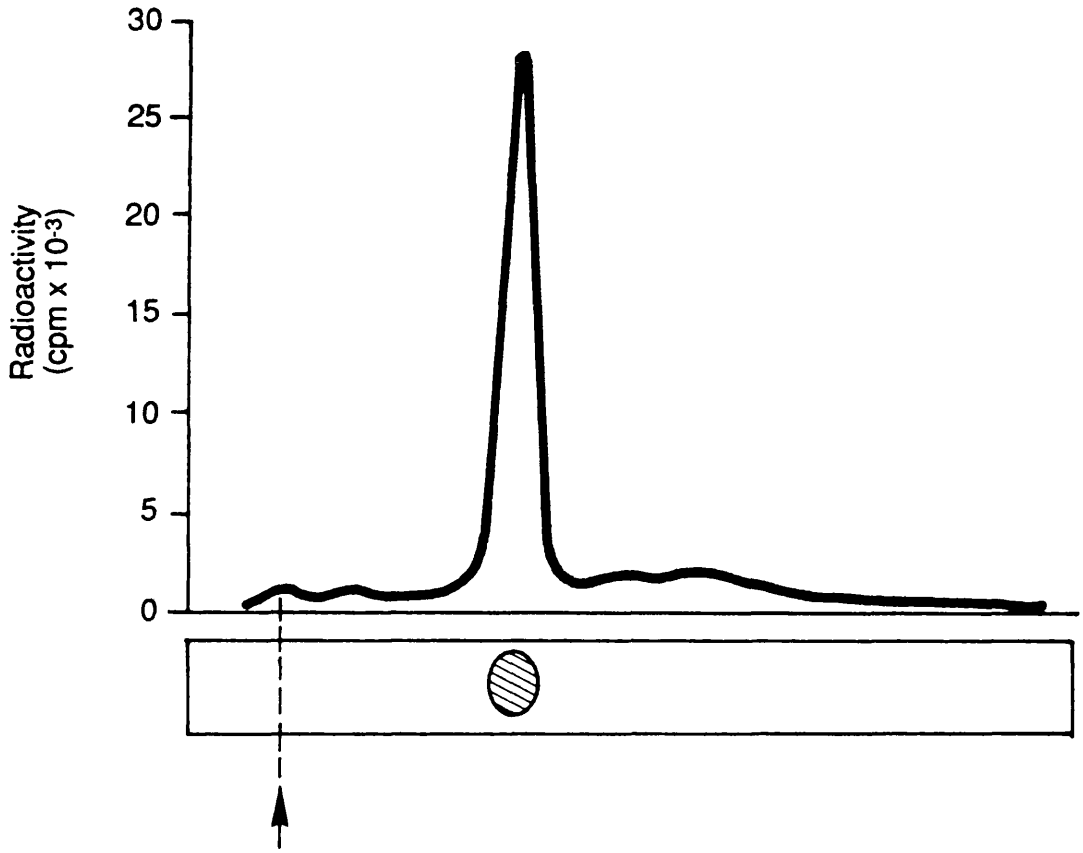

Radioactivity
(cpm x 10-3)
30
25
20
15
10
5
0

Fig. 2.9 Two-dimensional chromatogram of [22,23-^{3}H] dihydroazadirachtin

The figure represents a two dimensional TLC on silica gel of the radio-labelled product co-chromatographed with unlabelled dihydroazadirachtin carrier. The shading represents the presence of radio-activity. Arrows indicate the direction of development of the solvents as described previously.

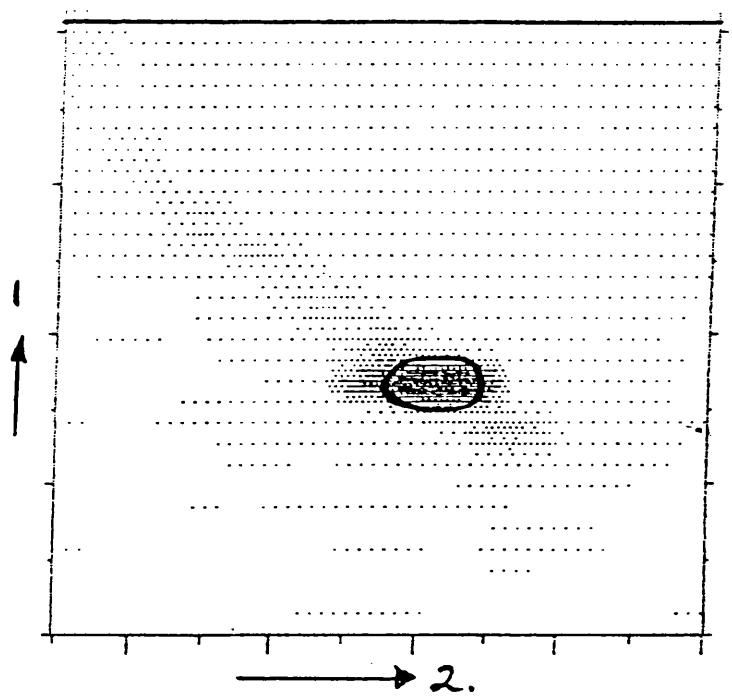

1
2.

Fig. 2.10. One-dimensional chromatography of [22,23-^{3}H]-dihydroazadirachtin.

The figure represents a chromatogram run as in figure 1. The upper trace of radio-activity was obtained by scanning as before, while the lower histogram was obtained by counting the radio-activity on 1cm segments of the chromatogram as previously described. As before, the arrow represents the point of application of the derivative.

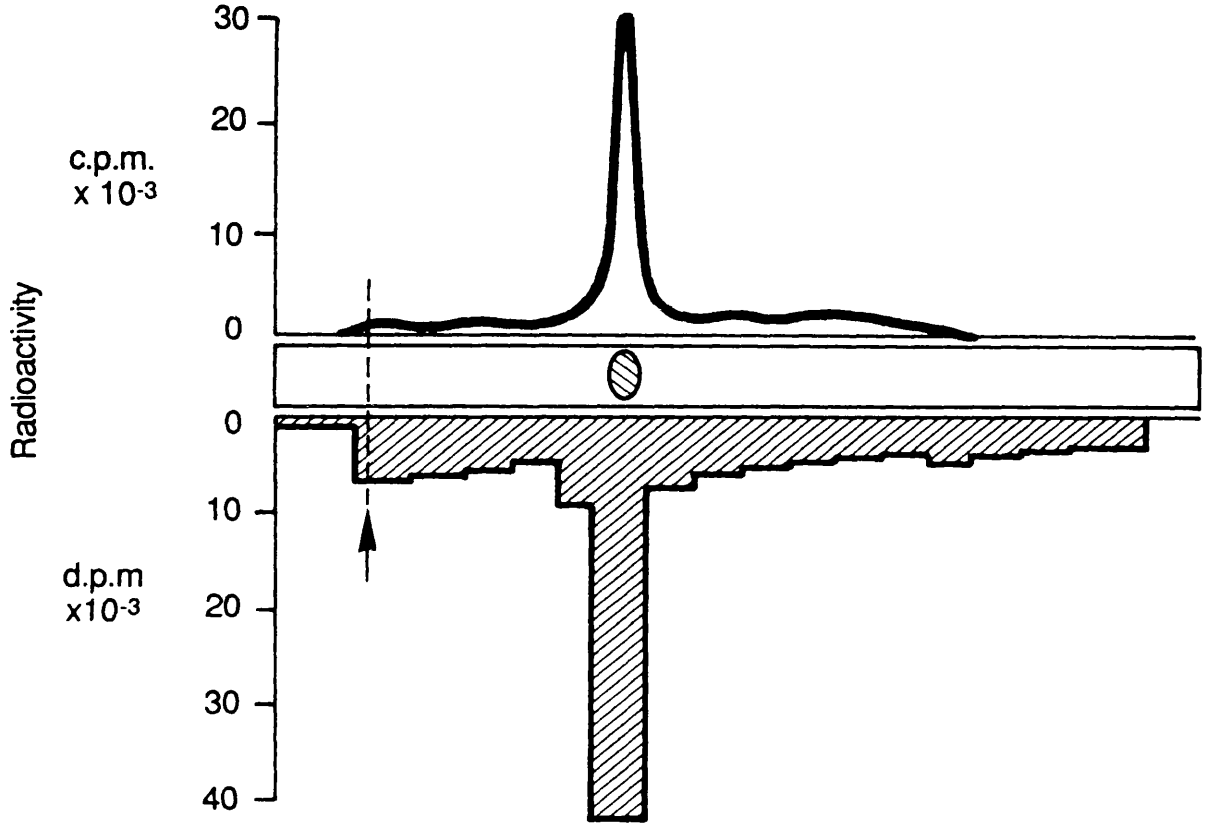
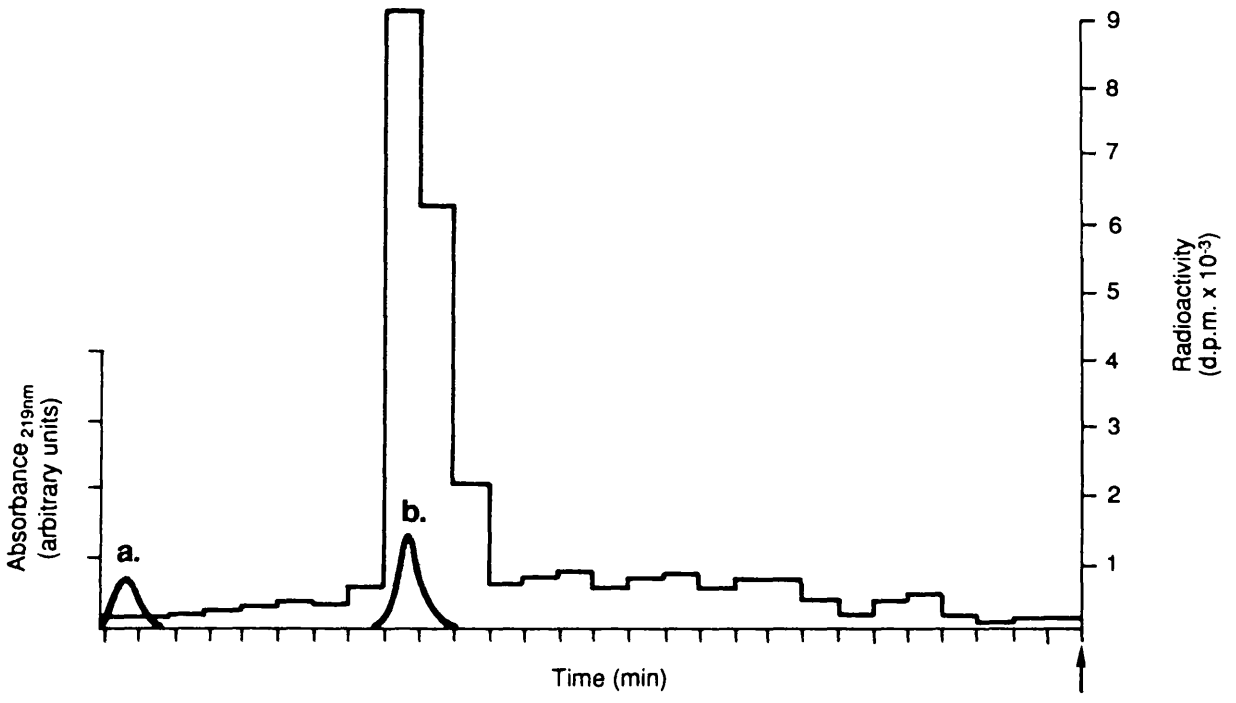

Radioactivity
c.p.m.
x 10-3
30
20
10
0
0
10
d.p.m
x10-3
20
30
40

Fig. 2.11. HPLC separation of azadirachtin and dihydroazadirachtin

The figure represents the co-chromatography of azadirachtin, tritiated and unlabelled dihydroazadirachtin, by reverse phase HPLC under the conditions previously described. The solvent was methanol/water 40/60 (v/v). 1ml samples of eluant were collected and their radio-activity estimated by scintillation counting. The arrow indicates the injection time of the mixture. Previous chromatography of pure compounds allows identification of (a) as azadirachtin and (b) as dihydroazadirachtin.


Absorbance 219nm
(arbitrary units)
a.
b.
Time (min)
Radioactivity
(d.p.m. x 10-3)
9
8
7
6
5
4
3
2
1

Another important consideration in the extraction and isolation process is the stability and shelf-life of the extractives and their formulations. It was shown that 30% of azadirachtin decomposed within 24 hours when left in chloroform (Zanno, et al. 1975) and therefore, the influence of the pH of the solvent or medium is critical in such cases. The effect of sunlight on the crude materials and extracts should also be borne in mind. It has been reported that, as exposure to sunlight increases, there is a rapid decrease in the antifeeding potency of azadirachtin (Stokes, et al. 1982). Benge (1988) lists genetic and environmental elements and maturity as factors which affect the azadirachtin content of seeds from individual trees. The ripeness of the seed at harvest is also imporant because, as ripening proceeds, the products formed are successively more highly oxidised. For this reason the major triterpenoid present varies from nimbin to salannin to azadirachtin as the seed ripens (Jones et al, 1988). The condition and duration of storage of the seed after harvest can also have a profound effect on azadirachtin content. Ermel et al. (1987) reported that the azadirachtin content of neem seeds fell to 10% of its original value when the seeds were stored at 100% relative humidity, and 60°C for 3 weeks. Schmutterer and Zebitz (1984) found that seeds which had been in storage for 18 months contained less methanol extractable material than those which had been stored for 4-6 months, though it was not clear which components of the seed were lost or reduced over time. The seeds used in the present extraction had been in storage, at room temperature, for approximately 5 months.

Isolation of azadirachtin is tedious, involving most of the following techniques: partition between solvents, column chromatography, preparative TLC and HPLC etc. Butterworth and Morgan (1968) isolated 1.51g of azadirachtin from 2 kg of seeds by extraction with ethanol, and partition between solvents followed by chromatography on floridin earth and finally by multiple elution preparative TLC. Zanno et al (1975) carried out two partitions of the ethanolic extract of the

seed kernel, first between ethyl acetate and water and then between methanol and hexane. The resultant polar mixture was twice chromatographed over silica gel using ether-acetone elution mixtures followed by chloroform-ethyl acetate elution mixtures. Pure material was then obtained by preparative TLC. A simplified procedure, introduced by Schroeder et al (1987), involved first defatting the neem seed kernels with hexane before ethanol extraction. The ethanolic extraction was partitioned subsequently between hexane and water to remove adhering fats, sugars, and proteins. The resultant residue in ethyl acetate was finally subjected to flash chromatography to yield azadirachtin of 70-80% purity. The USDA (United State Department of Agriculture) method (Uebel et al. (1979) for the large scale isolation of >90% pure azadirachtin involved extraction of defatted marc with acetone and washing of the acetone extract with hexane, and water and hexane, partitioning steps with 70:30 and 75:25 methanol: water and ether:acetone (25:75) followed by florosil column chromatography. The crude azadirachtin was finally purified by open reverse phase column chromatography employing phasebonded C-10 florosil with methanol : water (1:1) as eluting medium. Another procedure was introduced by Yamasaki et al (1986). Dichloroethane partition yielded an azadirachtin enriched fraction which was then subjected to sequential normal phase and reverse phase flash and preparative HPLC to give 98% pure azadirachtin with 29% overall efficiency.

The present work was carried out using a combination of all these methods which afforded >95% pure azadirachtin from neem seeds. The extraction of azadirachtin with methanol was more efficient when the ground neem seeds were first defatted with n-hexane. To ensure complete recovery of azadirachtin the defatted seeds were extracted four times with methanol at room temperature. The residue was purified another four-fold by partitioning between methanol and n-

hexane. It is possible that the de-oiled marc would possibly have been less compacted if the seed had been ground less finely.

The ethyl acetate extract was subjected to flash column chromatography to isolate the compounds because flash column chromatography is a rapid, inexpensive and easily performed technique with large sample capacity. The initial column chromatographic separation of the ethyl acetate soluble extract used a small range of eluting solvents which increased in polarity gradually and the fraction size was fairly large. As a result the separation was imprecise and some of the azadirachtin-containing fractions were very impure. These fractions were subjected to further flash chromatographic separation. With subsequent extracts it was found to be extremely advantageous to use shallow elution gradients throughout the purification procedure.

The thin layer chromatographic method of estimation the amounts of individual compounds such as azadirachtin, deacetyl salannin etc. can best be considered as semi-quantitative, its main limitation being the poor resolution of these compounds, eg: the R_f values of azadirachtin and deacetyl salannin are very close to each other. As a result, it was very difficult to estimate confidently the amount of azadirachtin co-occurring with its closely related analogues in crude extracts. To over come this problem the vanillin spray reagent was used to visualize azadirachtin on the TLC plate since it gave a characteristic colour reaction of red-brown changing to green.

Higher purity of azadirachtin can be achieved by preparative HPLC techniques. The purification of azadirachtin by flash chromatography followed by preparative HPLC had been reported (Yamasaki et al., 1986). This produced azadirachtin with a purity of >99% but it yielded of only 0.0056% (w/w). In the present study the yield of azadirachtin was 0.065% from Sri Lankan neem seed. This value is higher than from seed from Pakistan and Ghana (Nisbet, A.J.,1992).

The azadirachtin content of neem seed differs with geological location (Ermel et al., 1984). The highest yields of azadirachtin are not restricted to seed from a specific country, but come from individual trees of different geographical origin.

Confirmation of purity was carried out using HPLC analysis and NMR analysis. HPLC analysis is the best of most reliable method reported. Warthen et al (1984) employed a 10μ Radial-pack, μ- Bondapack C-18 column in a Z module radial compression separation system using methanol, water (1:1) with a flow rate of 2 ml / min and detection at 214 nm. Yamasaki et al (1986) employed two methods, one using a "Phenomenex" column, a solvent mixture consisting of acetonitrile : water (3:7) isocratically with a flow rate of 1ml min^{-1} and detector set at 219 nm and the other employing Alltech silica gel, isopropanol:hexane (1:3, 1ml min^{-1}) isocratically. Devakumar et al (1992) used acetonitrile:water (1:1, 1 ml min^{-1}) and an ODS chromosorb column for the analysis of nimbin, deacetyl nimbin and azadirachtin. Govindachari (1990) employed both methanol:water and acetonitrile:water eluants with an RP-18 column. The present work was carried out using a Spherisorb C-6 column and a solvent mixture consisting of methanol:water (1:1) with a flow rate of 1 ml min^{-1}. This system was chosen since it not only provided good separation of azadirachtin on reverse phase HPLC, but also because it did not interfere with the detection of azadirachtin and dihydroazadirachtin at 218 nm.

The structure of azadirachtin (**1**) was elucidated by analysis of NMR spectra and finally by X-ray crystallography of a derived product (Bilton et al., 1987) . Azadirachtin H was isolated from an extract of neem seed kernals by preparative HPLC and its structure was assigned on the basis of ^{1}H and ^{13}C NMR (Govindachari, et al., 1992). The multiplicities of all carbon signals were assigned by DEPT experiments. The proton-proton connectivities were established by a ^{1}H-^{1}H COSY spectrum and decoupling experiments. It is obvious from the NMR data

that azadirachtin H differed from azadirachtin A by the substitution of a hydrogen for the carbomethoxy group at C-11. H-11, appearing at δ_H 5.41 (d, J= 4.4 Hz), was found to be coupled to H-9 at δ_H 3.19 (d, J = 4.4Hz) (COSY). The carbomethoxy group linked to C-11 in azadirachtin A appears at δ_H 3.68 (s), and is absent in the spectrum of azadirachtin H.

The results shown in Fig. 2.11 suggest that the only labelled compound present is in fact dihydroazadirachtin . Some back-ground activity appeared in the chromatograms, but examination of this suggests that it is not associated with a specific compound.

Estimation, by means of HPLC, of the amount of the tritiated compound obtained, indicates a total quantity of 9.4 mg (13.3 μmol). As the total volume is 40 ml, this means of concentration of 235 μg/ml. This indicates a yield of slightly less than 50% of the 20 mg used by Amersham Ltd. Estimates of the amount of radio activity in dpm indicates a specific activity of 27 Ci/mmol. This is a somewhat higher specific activity than that obtained by Rembold et al. (1988).

CHAPTER 3

UPTAKE, RETENTION, METABOLISM AND EXCRETION OF [22,23-$^{3}H_2$] DIHYDROAZADIRACHTIN IN *SCHISTOCERCA GREGARIA*.

3.1. INTRODUCTION

Azadirachtin is the predominant growth-inhibiting neem compound and the locust *Schistocerca gregaria* is particularly sensitive to it. Various hypotheses proposed to explain the action of azadirachtin on insect growth and development include a disruption of neuroendocrine control of moulting hormone synthesis (Sieber et al., 1983), a juvenile hormone effect (Malczewska et al., 1988), an inhibition of eclosion hormone release (Schluter et al., 1985) and an inhibition of chitin synthetase activity (Schmutterer, 1988). It has been also suggested that azadirachtin may be functioning as a moulting hormone analogue (Ruscoe, 1972). The similarities observed between azadirachtin and insect moulting hormones (ecdysteroids), both in their stereo-structures (Taylor, 1987; Turner et al., 1987) and in their effects on insects (Kubo et al., 1986) lend support to this suggestion.

Although the general neuroendocrine effect of azadirachtin is well established, very little is known about its cellular mode of action. Initial studies carried out on the tissue-specific incorporation of azadirachtin using tritium labelled 22,23-dihydroazadirachtin show that some 75% of labelled material is rapidly excreted from the haemolymph within 24 hours of injection leaving a residue which is permanently bound to different tissue components (Rembold et al., 1988; Garcia et al., 1989; Barnby et al., 1989b). Dry mount autoradiography of tissues from 8-day-old adult female *L. migratoria* injected with [$^{3}H_2$]-dihydroazadirachtin 5 days previously revealed specific and dense labelling of the corpora cardiaca and neurilemma and the brain (Subrahmanyam et al., 1989a) and of the Malpighian tubules (Rembold et al., 1988). Autoradiographs showed the neurosecretory axons of the corpora cardiaca and the Malpighian tubule cells to be heavily labelled. The latter were specifically labelled in the region of basal infoldings of the cells and round the nuclear membrane. The cell cytoplasm, apical and tubule lumen were not

labelled. Extraction of labelled material from the Malpighian tubules showed it to be mainly in its original form so suggesting the presence of high affinity binding sites at the basal infolding of the Malpighian tubule excretory cells (Rembold et al., 1988).

A fundamental assumption is that some insect tissues possess specific receptors for azadirachtin (Kauser et al, 1984). Evidence that close analogues of the compound have different biological potencies support this contention (Jacobson, 1988).

To investigate this hypothesis more exactly, a radiolabelled tracer is required, and Rembold et al.,(1983) introduced the use of [22,23-$^{3}H_2$] dihydroazadirachtin(2). Although the dihydro derivative is structurally different from azadirachtin (Fig. 3.6), current evidence suggests that its biological activity is the same (Rembold et al, 1983). Rembold and his co-workers (Rembold et al, 1983, 1988; Subramanyam et al, 1989a and Sieber et al, 1983) have done preliminary work with both locusts and other insects which has indicated that specific tissues, eg Malpighian tubules, do have differential affinities for tritiated dihydroazadirachtin.

The aim of the present work was to confirm and extend the previously reported results, and in particular to try to establish, by the use of unlabelled analogues, the specificity of the uptake of dihydroazadirachtin into locust tissues, as a preliminary to attempting more exact binding studies on identified tissue fractions.

A secondary objective was to examine uptake after topical application of the radio-labelled compound, and so determine if the route of application determined tissue distribution.

Finally, as the effects of azadirachtin and its analogues are often apparent over periods of days rather than minutes, the rate and extent of metabolism

is obviously of importance. A preliminary study was made of the metabolites of the [22,23- $^{3}H_2$]-dihydroazadirachtin, as previous results are ambiguous (Rembold et al, 1983 and 1988).

3. 2. METHODS AND MATERIALS

3.2.1. Insects

Schistocerca gregaria were purchased from Blades Biological, Edenbridge, Kent, and were maintained under laboratory conditions in metal cages at a temperature of 28°-30°c, approximately 40% relative humidity, and a light /dark cycle of 12 hours. The insects were fed on fresh washed spring cabbage leaves. Fresh tap water was also supplied. Cages were cleaned daily. Adults and larvae of male and female locusts were used for the experiments.

3. 2. 2. Preparation of azadirachtin

Azadirachtin was extracted and purified as described in Chapter 2.

3. 2 . 3. Hydrogenation of azadirachtin

22,23-Dihydroazadirachtin (**3**) was prepared as described in Chapter 2.

3. 2. 4. Application of [22,23-$^{3}H_2$]-dihydroazadirachtin.

3. 2. 4. 1. Injection

The tracer was initially dissolved in ethanol, which was then diluted with distilled water to 10% v/v ethanol/water to give a final concentration of 336μmol/l (9.1mCi/ml). 3μl (0.6nmol) of above solution was injected in to each locust. Injections were made through the abdominal intersegmental membrane using a

Hamilton 10 µl syringe. Individual locusts were then kept in separate beakers for periods of up to 7 days before being killed. During this time they were fed on fresh cabbage leaves. When it was required to examine the excretion for radio-activity, the faeces of each locust were collected at the end of each 24 hour period. It was noticed that part of the excreta was liquid, which was absorbed by the filter-papers lining the beakers, and this was also radio-active. Care was taken to include this material in the total excretion of the locust.

To discover if a large excess of unlabelled dihydroazadirachtin could displace the label from the locust tissues after several days, 10^3 times the tracer dose was injected 48 h after the injection of tritiated dihydroazadirachtin. Any subsequent faecal pellets were examined for the presence of radio-activity as below.

3. 2. 4. 2. Topical application

0.6nmol of [22, 23 -$^3H_2$]-dihydroazadirachtin was applied in acetone (3µl) as a streak along the ventral thoracic surface of each locust. Distribution of radioactivity was examined in different tissues of the locusts 1 day and 7 days after injection, after carefully washing the cuticle of the insect to remove the tracer still present on the outer surface of the cuticle. The methanol washings were pooled and the radio-activity estimated.

3. 2. 4. 3. Effect of a large excess of unlabelled analogues.

Different concentrations of unlabelled analogues (up to 10^4 molar greater than the tracer concentration) were introduced into the haemolymph of the locusts as described for the tracer, and the tracer dose of tritiated dihydroazadirachtin was injected 5 min after injection of the unlabelled compounds. The rate of

disappearance of radioactivity from the haemolymph was then followed as described below.

3. 2. 5. Tissue sampling

3. 2. 5. 1. General survey.

The alimentary system of the locust was removed from the rest of the body, and carefully rinsed through with saline, before being divided into crop, hepatic caeca, Malpighian tubules and hind gut. The rest of the body was also cut into six pieces: head, anterior and posterior thorax, and anterior, mid- and posterior abdomen. Most of these correspond with the divisions of the gut.

3. 2. 5. 2. Specific tissues

Individual tissues were dissected, and frozen in liquid N_2, before grinding in a mortar precooled with liquid N_2. Samples of the individual tissues were homogenised with a loose-fitting teflon homogeniser in 1ml of methanol to extract the radiolabel and precipitate the tissue protein, which was then removed by centrifugation for 5min at 10,000g. Samples of the supernatant were taken for estimation of the radioactivity by scintillation counting.

The protein pellet was redissolved in 0.1M NaOH containing 0.1% w/v SDS overnight at 30°C. It was then estimated by the method of Lowry et al (1951), against a standard of bovine serum albumin, and the specific activity of the labelled compound in the tissue was estimated with reference to the protein content.

3. 2. 5. 3. Haemolymph

Samples of haemolymph (1µl) were taken out from the base of the hind coxa of the locust using 5µl micropipettes.

3. 2. 5. 4. Faeces

The faeces were air dried overnight, and the daily samples were ground separately with mortar and pestle. Each sample of faeces was then extracted with 2ml of methanol as described above for frozen tissues. Samples of the methanol extract were used for scintillation counting and chromatography.

Pieces of the filter paper from the beakers, which clearly showed staining due to liquid excretions, were cut out and the absorbed radio-activity estimated by scintillation counting.

3. 2. 5. 5. Primary urine.

In order to determine if dihydroazadirachtin was actively secreted by the Malpighian tubules, the following preparation was set up *in vitro*. Locusts which had been starved for 12 h to empty the gut and eliminate diuretic effects, were dissected under saline, and the gut ligatured immediately anterior to the point of entry of the tubules. 5µl graduated micropipetes, pretreated with 2% dichlorodimethylsilane (Fisons Scientific Equipment, Loughborough), were then inserted into the gut immediately posterior to the Malpighian tubules, and ligatured in place. The preparation was then placed in 2ml of locust saline containing 10mM glucose as energy source, and 0.6 nmol tritiated dihydroazadirachtin. The saline was maintained at a constant temperature of 30 ^{o}C. Secretion of water was then stimulated by addition of 10^{-3} M c-AMP. Once the pipette had filled with secreted fluid, samples were taken for scintillation counting.

3. 2. 6. Estimation of haemocoel volume and mixing time

[^{3}H]-Inulin was used as a space marker to estimate the volume of haemolymph and also the time required for mixing of an injected tracer in the

haemolymph (Loughton et al, 1969). A sample of ^{3}H-labelled inulin (0.72μCi) was injected into each locust by the same route as the tritiated dihydroazadirachtin, and haemolymph samples (1μl) were taken as described and the radio-activity estimated by scintillation counting, until the radioactivity in the haemolymph became constant. As a result of these preliminary studies, it was found that complete mixing of the marker required a minimum of 5 minutes. The haemolymph volume of adult locust was found to be 500± 25μl, and this figure was used to determined the maximum theoretical concentration of the tracer dose of tritiated dihydroazadirachtin. This was calculated as 1.2×10^{-6}M.

3. 2. 7. Metabolism of dihydroazadirachtin

Methanol extracts of faeces and dissected tissues were individually chromatographed on a silica TLC plate, with ethyl acetate as solvent. After development, the radioactivity was measured using both the radiochromatoscanner and scintillation counting. Co-chromatography of unlabelled dihydroazadirachtin allowed location of the tritiated derivative by U.V. absorbtion.

3. 2. 8. Estimation of radio-activity

3. 2. 8. 1. Tissues

Sections of gut and cuticle were placed in 2ml of 25% w/v KOH, and boiled until the soft tissues had dissolved. Duplicate samples of this (2μl) were taken for scintillation counting.

3. 2. 8. 2. Haemolymph

Samples of haemolymph (1μl) were directly added to the scintillation fluid and radioactivity was measured.

3. 2. 8. 3. Thin layer chromatography

After development of the plates and location of identifiable compounds, the chromatogram was divided in to 1cm strips, the silica from which was scraped into individual scintillation vials. Ecoscent-A (National Diagnostics, Atlanta, Ga, USA) was used as scintillation fluid. 4ml aliquots were poured into each vial, and the radioactivity was measured using the Wallac 1409/11 liquid scintillation counter programmed for quench correction.

3. 3. RESULTS

3. 3. 1. Excretion of injected radio-activity

The excretory profile of injected [22,23-$^{3}H_2$]-dihydroazadirachtin is shown in Fig. 3.1. Almost half of the applied radioactivity was excreted during the first two days, almost all of that in the first 24 hours, and the remainder was retained in the body. Very little radio-activity was excreted after 48 hours, although there was a small, constant, daily excretion. It was possible to account for >95% of the total radio-activity, even after 7 days.

Injection of a 1000-fold excess of unlabelled dihydroazadirachtin after 48 hours caused only a small and variable excretion of labelled material averaging 5% of the total injected. Due to the antifeedant and toxic effects of the compound, no futher excreta were produced.

There was no evidence that the tritiated dihydroazadirachtin was actively concentrated in the primary urine produced by the Malpighian tubule preparation. The results obtained over a period of one hour were variable, but 4 separate preparations indicated an average concentration of radiolabel in the urine only 13 $\pm$ 7% of that in the surrounding medium.

3. 3. 2. Retention of radioactivity in the tissues of the locust

Fig. 3.2 shows the distribution of [22,23-$^3H_2$]-dihydroazadirachtin in the adults and larvae of *Schistocerca gregaria* 1 day and 7 days after injection. A crude separation of the body into gut and carcase, indicated that 45% of the injected label was in the former. Subdivision of the gut showed that the portion including the Malpighian tubules contained the largest amount of radio-activity (12-15%), with a smaller amount in the crop (8-10%). The hind gut contained little of the total tracer. It was found that if the hepatic caeca were sampled without opening and washing each internally, 30-40% of the total retained activity was present in this section. If, however, they were carefully rinsed out, the activity in this tissue fell to a fraction similar to that of the hind gut, about 5% of the total.

Initial attempts to subdivide the rest of the body in to segments failed to show any differences in labelling comparable to that shown by the gut, and so the results were simply combined to give a total for the "rest of the body". Little difference was found between 1 and 7 days in the general pattern of labelling.

The major difference between adult and 5th instar larvae was that in the latter almost 30% of the activity was found in the section containing the Malpighian tubules. This was significantly greater than the adults.

3. 3. 3. Topical application

The absorption of [22,23-$^3H_2$]-dihydroazadirachtin into the locust was initially slow when it was applied topically, and it was only after 24 hours that tracer could be detected in the haemolymph. Most of the radio-activity was recovered in the methanol washings of the cuticle, although it was only possible to account for 75% of the total applied material. After 7 days, 17% of the applied dose had penetrated into the body, and 2.4% of applied dose was extracted from the faeces over this period. The pattern of retention of radio-activity was different after

topical application,with very little present in the Malpighian tubules, and 70% remaining in the "rest of the body".

3. 3. 4. Uptake of label by identified tissues

When the retained radio-activity in individual tissues was related to protein to give some measure of specific uptake of label, (Table 3.1), Malpighian tubules were shown to retain a high concentration of the tracer, but that both testis and ovary were equally or more highly labelled.

In general, the tissue activity in the 5th instar larvae was similar to that of the adult, but one clear difference was that the nervous tissue (brain, corpus cardiacum, and ventral ganglia) of the larvae showed a high level of labelling, while the corresponding tissues in the adult were almost free of label. There was no evidence for a high level of labelling of the prothoracic glands in the 5th instar larvae, but the tiny quantity of tissue available made it impossible to produce exact quantitative results.

3.3.5. Uptake of [22,23-$^{3}H_2$]-dihydroazadirachtin from the haemolymph.

When a tracer amount of tritiated dihydroazadirachtin was injected by itself into the haemolymph, it was removed at great speed. Preliminary studies with radio-labelled inulin, injected and sampled by the same routes as the dihydroazadirachtin, showed that this space marker was completely distributed in the haemolymph 5 min after injection. Two minutes after its injection, only 14% of the total amount of labelled dihydro-azadirachtin injected remained in the haemolymph, and this fell almost to zero after 30 minutes.

Fig. 3.1. Excretion of radio-activity from the locust *S. gregaria* after injection of a tracer dose of ^{3}H dihydroazadirachtin.

Results are the means from 5 locusts, each of which received 0.6 nmol of tritiated dihydroazadirachtin with a specific activity of 27 $Ci.mmol^{-1}$, and thereafter kept separately with free access to fresh food and water. Bars indicate the standard deviation from the mean. In a separate experiment, 3 locusts received by injection a 1000 fold greater amount of unlabelled dihydroazadirachtin 48 h after the tracer (*).

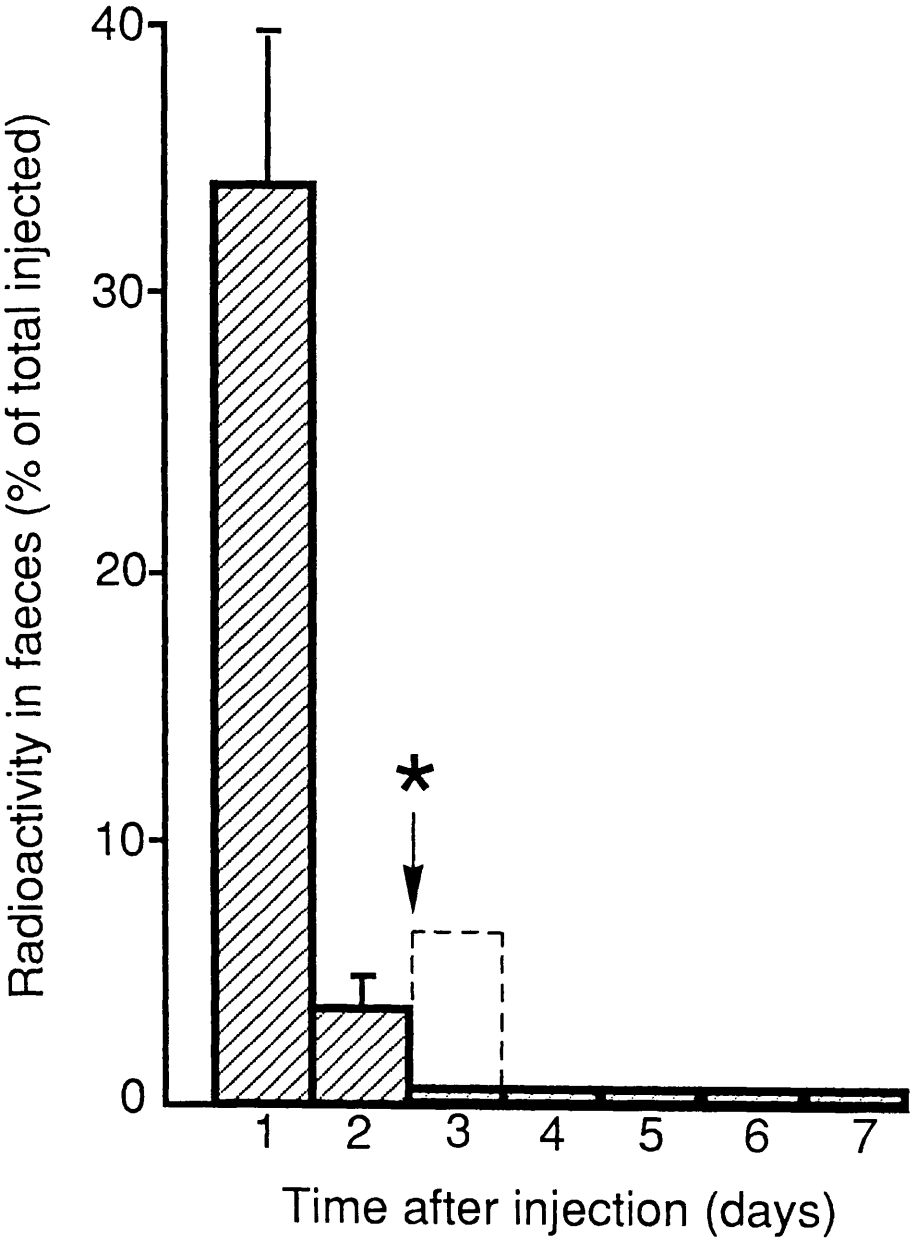
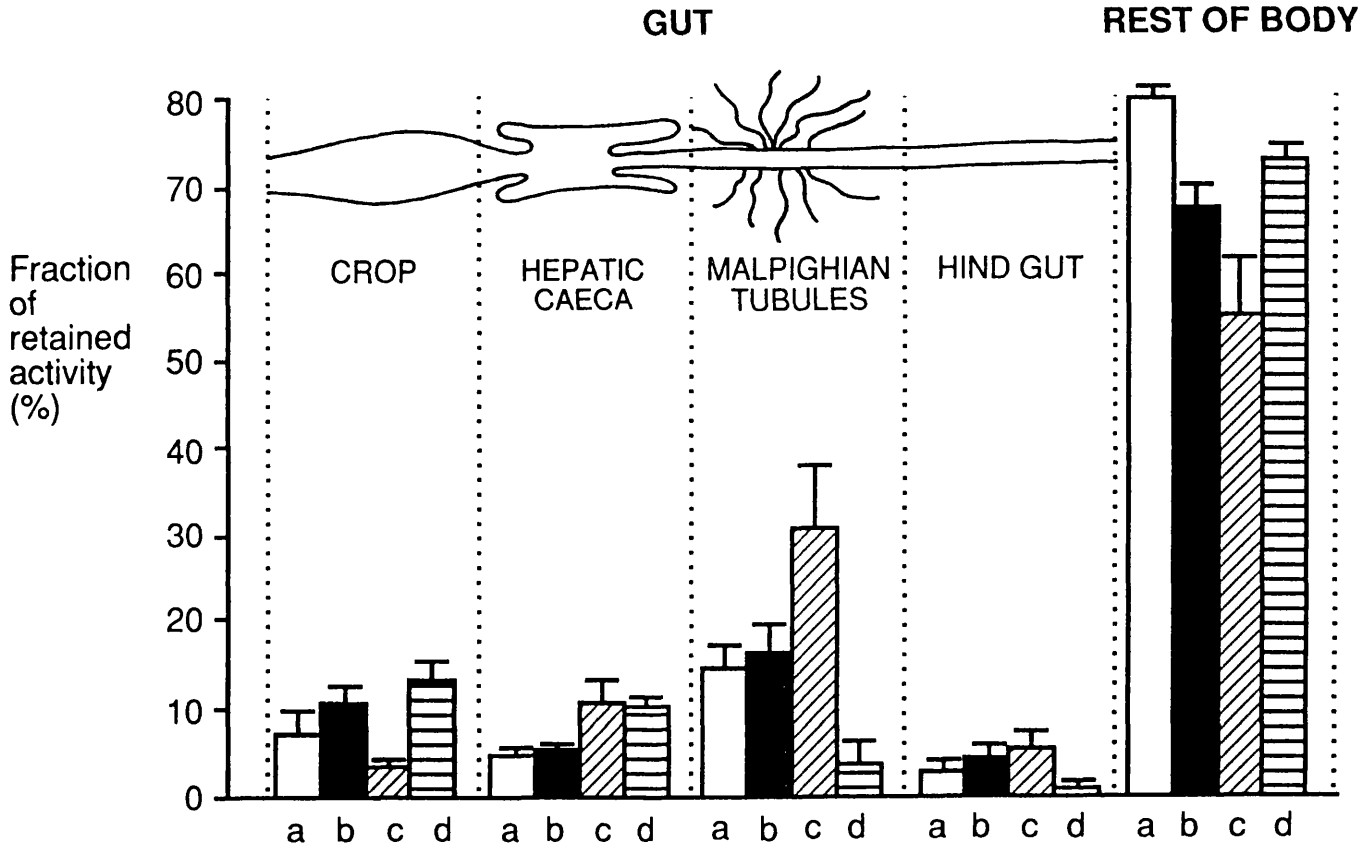

40
30
20
10
0
Radioactivity in faeces (% of total injected)
*
1
2
3
4
5
6
7
Time after injection (days)

Fig. 3.2. Distribution of retained radio-activity in the intestinal and other tissues of the locust *Sch. gregaria*, after injection or topical application of a dose of ^{3}H dihydroazadirachtin.

The results are the means (and S.D.) for the complete tissues indicated. The tracer was administered either by injection to adult insects and the tissues removed after a) 1 day, and b) 7days, and c) juvenile (5th instar) insects, or d) by topical application to the ventral side of the abdomen , before removing the tissues after 7 days. Results are from 5 locusts in each case. The drawing of the alimentary canal is purely diagrammatic.


GUT
REST OF BODY
Fraction of retained activity (%)
80
70
60
50
40
30
20
10
0
CROP
HEPATIC CAECA
MALPIGHIAN TUBULES
HIND GUT
a b c d a b c d a b c d a b c d a b c d

The results in Figs. 3.3 and 3.4 show the effects on this uptake of a large excess of unlabelled dihydroazadirachtin, and of lowered temperature.. A 10^3 higher molar concentration of the unlabelled compound considerably reduced the rate of uptake of label, while $4x10^3$ excess eliminated 70-80% of the uptake over the time period studied. Further increase of the concentration of the unlabelled material to 10^4 excess produced the same results as $4x10^3$.

Table 3.1. Persistent binding of $^3H_2$-dihydroazadirachtin to specific tissues of the locust *Sch.gregaria*.

Tissue	Radiolabelled dihydroazadirachtin bound (pmol.mg^{-1} protein)	
	Adult	Larva
Crop	0.2±0.002	0.3±0.01
Hepatic caeca	4.42±0.32	3.97±0.52
Malpighian tubules	2.9±0.65	2.7±0.51
Ovary	3.5±0.81	-
Testis	2.9±0.72	-
Flight muscles	0	0
Fat body	1.6±0.20	1.3±0.40
Nerve tissue	0	5.9±1.50
Rest of the body	1.4±0.01	1.6±0.22

A tracer dose of tritiated dihydroazadirachtin (0.6nmol) was injected into the haemolymph of the insects: adults of both sexes and 5th instar juveniles. After seven days, the tissues were dissected and homogenised in 90% methanol to extract the labelled material and precipitate the protein, which was then redissolved before estimation. The results are the means (±SD) of 5 insects.
"Rest of the body" refers to the fat body, musculature and cuticle.

Fig. 3.3 Effect of low temperature on the disappearance of $^{3}H_2$-dihydroazadirachtin from the haemolymph of the locust.

The tracer dose, 0.6nmol of tritiated dihydroazadirachtin, was injected into the haemolymph at 0 time and samples taken as described. The points are the mean of 5 locusts, and the vertical bars indicate the standard deviation. The solid bar indicates the time during which the locusts were held in ice. For comparison, the data for the disappearance of tracer at room temperature (25^{o}C), are also shown (----).

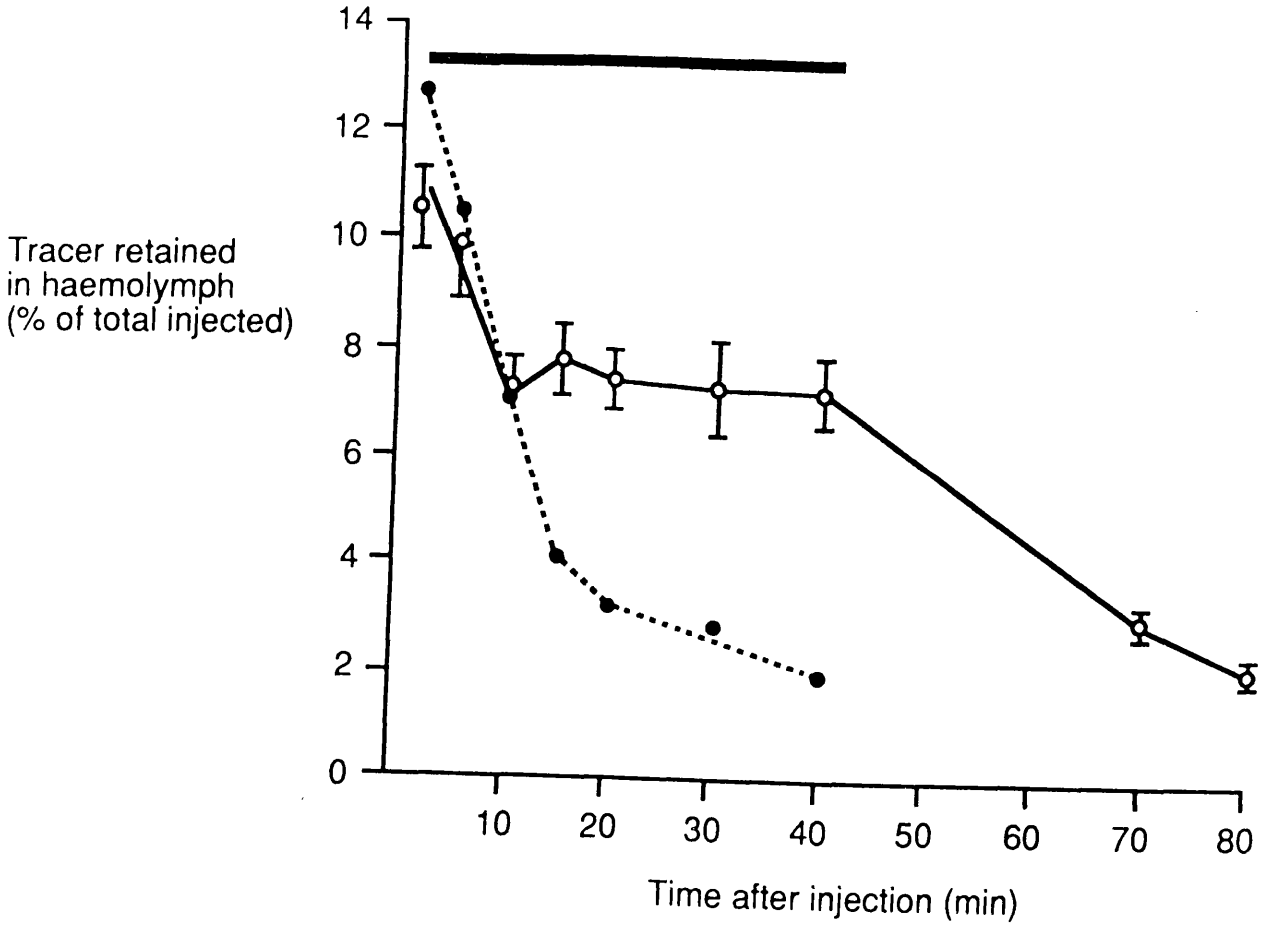
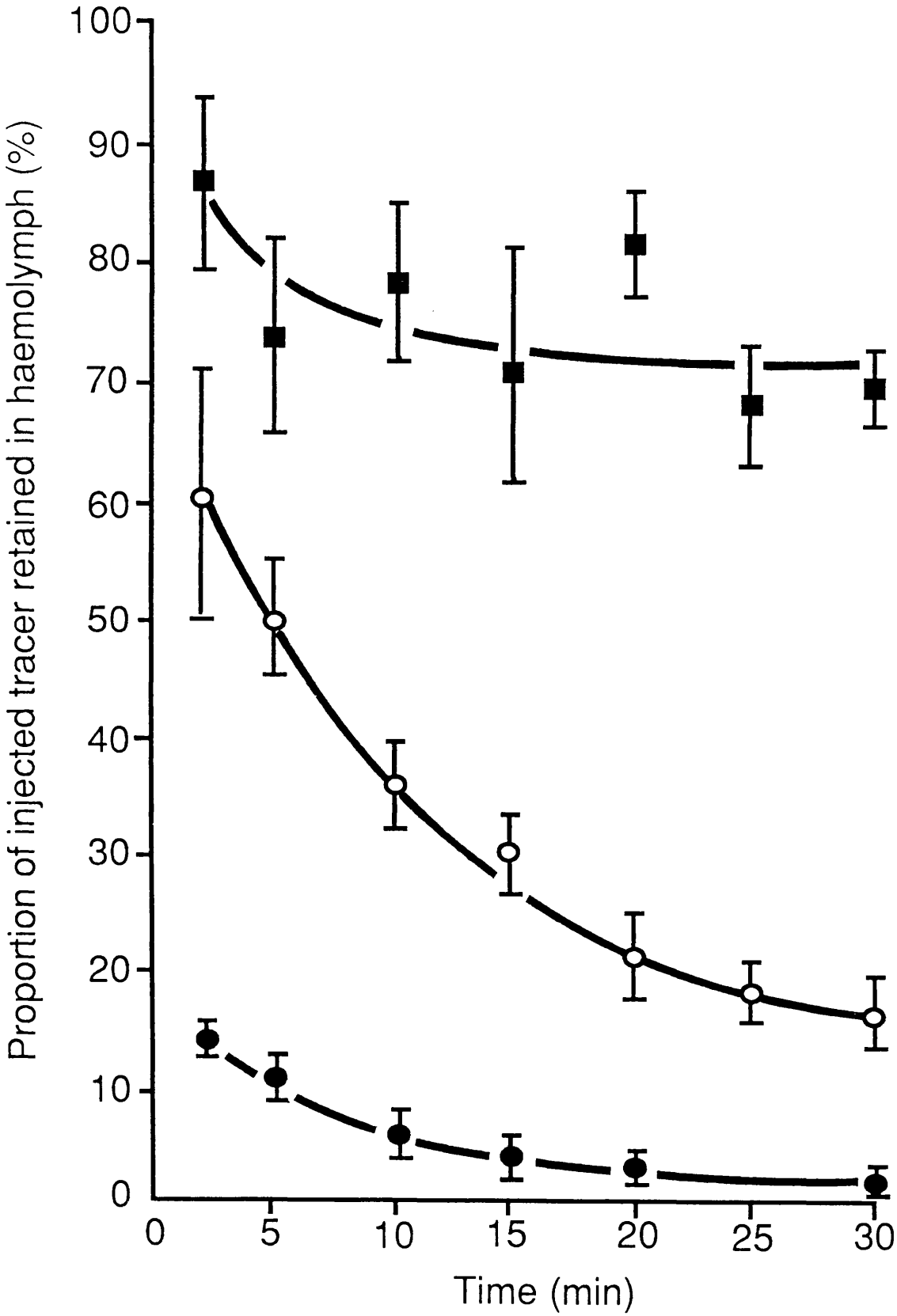

Tracer retained
in haemolymph
(% of total injected)
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
Time after injection (min)

Fig. 3.4. Clearance of $^{3}H_{2}$-dihydroazadirachtin from the locust haemolymph.

A tracer dose of 0.6nmol of tritiated dihydroazadirachtin was injected at zero time either alone(●), or in the presence of 1000-fold (○) and 4000-fold (■) excess concentrations of unlabelled dihydroazadirachtin. The results are the means (±SD) from 5 individual adult locusts.


Proportion of injected tracer retained in haemolymph (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (min)

Placing the locusts in ice after the injection of the tracer dose of tritiated dihydro compound eliminated much of the uptake from the haemolymph. When the locusts were restored to normal temperature, the uptake resumed.

In Fig. 3.5 are shown the results of injecting large excesses of the parent compound (**3**), azadirachtin (**1**), 3-deacetylazadirachtin (**4**), 23-ethoxy-22,23-dihydroazadirachtin (**5**) and 3-deacetylsalannin (**7**) (Fig. 3.6). While azadirachtin inhibited the uptake of the tritiated dihydroazadirachtin it was clearly less effective than equal concentrations of unlabelled dihydroazadirachtin over the 30min period studied. 3-Deacetylazadirachtin had an inhibitory effect similar to the dihydroazadirachtin, as did 23-ethoxy-22-bromoazadirachtin(**6**), while 3-deacetylsalannin (**4**) had no inhibitory effect at all.

In Fig 3.7 are shown the effects of 1000 fold concentrations of the decalin (**9**) and furanoid ring (**8**) structures on uptake of the tracer. The two curves were vitually identical, and so only one is shown. The figures in Table 3.2 indicate that the inhibitory effect of the partial structures is similar to that of dihydroazadirachtin.

The shape of the curves presented in the figures suggested that the uptake of the labelled dihydroazadirachtin followed an exponential form, and this was confirmed when the results were plotted in a semi-logarithmic form (Fig 3.8). No results can be presented for the period before the 2 min sample, and the theoretical rates of uptake shown are presented as based on instantaneous complete mixing of the injected tracer. Nevertheless, it is clear that addition of an excess of various analogues affects these theoretical rates. After 2 min the data indicate that two mechanisms of uptake may be distinguished, with different rate constants. The results suggest that these different uptake processes may respond differentially to inhibition by analogues of the labelled compound.

As the uptake into as yet undefined pools was clearly heterogeneous, two points were chosen in the time course , 5 and 20 min, and the extent of inhibition under various conditions were compared (Table 3.2). These results give exact figures to the extent of inhibition of uptake and suggest that there are mechanisms which differ in their affinities for the compounds used. These differences seem to vary with time after injection. For example, while azadirachtin and dihydroazadirachtin were equally effective in inhibiting uptake at 5 min this was not true at 20 min. 23-Ethoxydihydroazadirachtin (**5**)proved to the most effective inhibitor of initial uptake, but by 20 minutes this effect was no longer apparent.

3. 3. 6. Metabolism of injected [22,23-$^3H_2$] dihydroazadirachtin.

Fig 3.9 shows the distribution of radioactivity, resolved by TLC, in the methanol extract of the faecal pellet after 24 hours. Some of the tritium appears in a polar compound (or compounds) which do not move from the origin. It is clear that metabolism of dihydroazadirachtin does take place in quite a short time. The proportion, however, of the polar compound to the original in the faeces did not change over the 7 days, and the results of extracting the radio label from individual tissues at the end of 7 days showed that while there was considerable sign of metabolism in fat body and crop, the label in the other tissues was almost unchanged (Table 3.3).

Table 3.2. The effect of possible competitors on the removal of a tracer dose of $^{3}H_2$-dihydroazadirachtin from the haemolymph of the locust *Sch. gregaria*.

Compound	Amount administered	Radio-activity removed from haemolymph (% of total)		Reduction of uptake (% of max.)	
		5min	20min	5min	20min
Control*	(1)	88±2	94±1	0	0
Dihydroazadirachtin (**3**)	$1x10^3$	50±6	78±4	43	17
Dihydroazadirachtin (**3**)	$4x10^3$	20±10	25±6	77	72
Azadirachtin (**1**)	$4x10^3$	25±10	56±5	72	40
Azadirachtin (**1**)	$10x10^3$	23±12	53±6	74	43
3-Deacetylazadirachtin (**4**)	10^3	50±7	65±3	43	31
23-Ethoxy-22,23- (**5**) dihydroazadirachtin	10^3	70±10	68±8	77	28
22-Bromo-23-Ethoxy (**6**) dihydroazadirachtin	10^3	50±4	77±3	43	17
3-Deacetylsalannin (**7**)	10^3	89±2	92±2	0	2
Decalin ring portion (**8**)	10^3	-	79±3	-	16
Furanoid ring (**9**) portion	10^3	60±4	84±3	32	11

* The control consisted only of the tracer amount of tritiated dihydroazadirachtin (0.6nmol), which gave an average maximum concentration of 1.2×10^{-6}M in the haemolymph. The concentration of the unlabelled competitors relate to this concentration in molar terms. The large excess of these was injected into the haemolymph 5min before the tracer.

All rates of uptakes are related that of the tracer dose which is taken as the maximum.The results are taken from the curves in Figs. 5,6, and 7. Statistical significance is indicated in the last two columns, different letters indicating significant differences ($P<0.01$).

Fig. 3.5 Clearance of $^{3}H_2$-dihydroazadirachtin from the locust haemolymph in the presence of structural analogues.

The tracer dose of tritiated dihydroazadirachtin was injected in the presence of 1000 fold concentrations of 3- deacetylsalannin (O), 4000 and 10,000 fold concentrations of azadirachtin (■) and 1000 fold concentration of 23- ethoxy -22,23 -dihydroazadirachtin (△), 1000 fold concentration of 3- deacetylazadirachtin (□). The results are the means (± SD) from 5 individual locusts. The results for the tracer alone (---) are included for comparison.

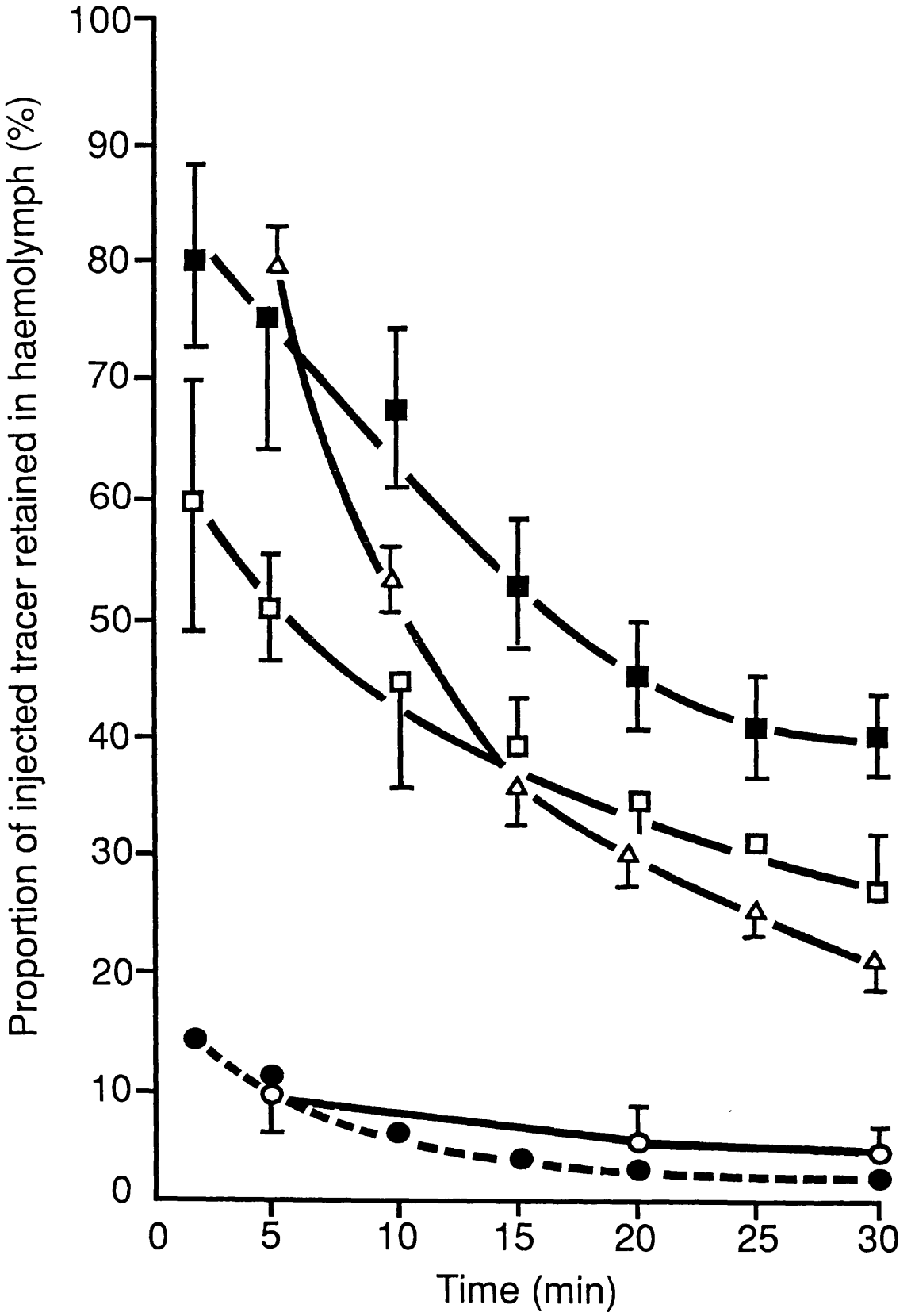
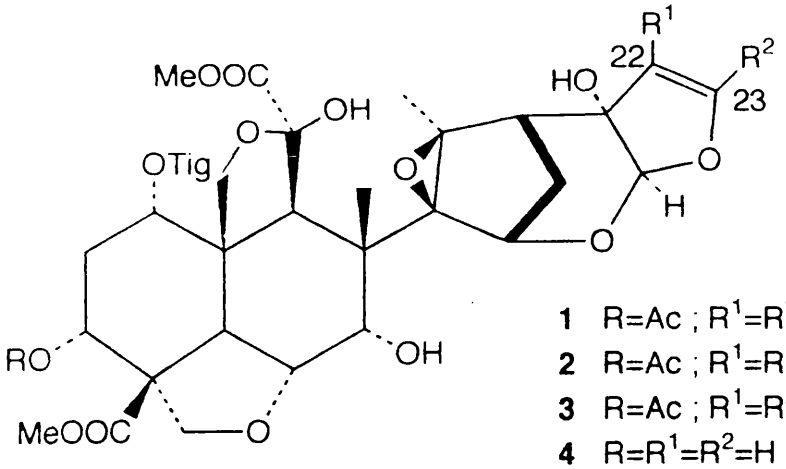
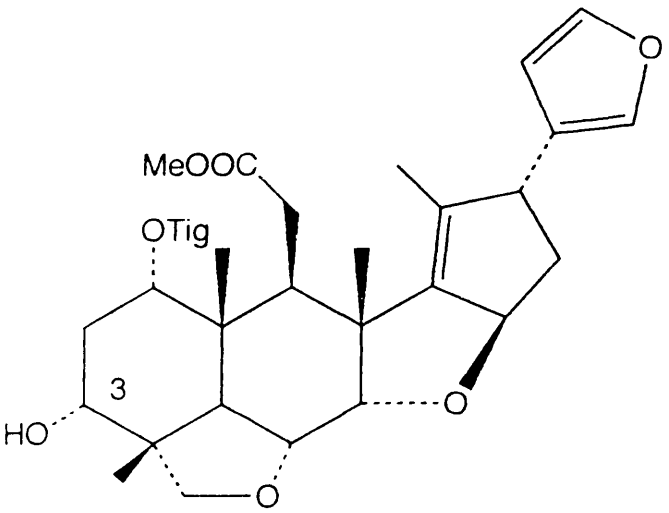

Proportion of injected tracer retained in haemolymph (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (min)

Fig. 3.6 Structure of azadirachtin and close structural analogues.

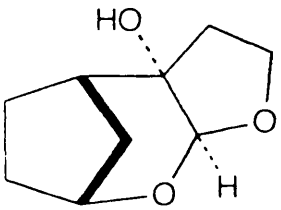
1. Azadirachtin **2.** [22,23-^{3}H] Dihydroazadirachtin
3. 22,23-Dihydroazadirachtin **4.** 3-Deacetylazadirachtin
5. 23-Ethoxy-22,23- dihydroazadirachtin
6. 22 Bromo-23-ethoxydihydroazadirachtin **7.** 3-Deacetylsalannin
8. Decalin ring **9.** Furanoid ring



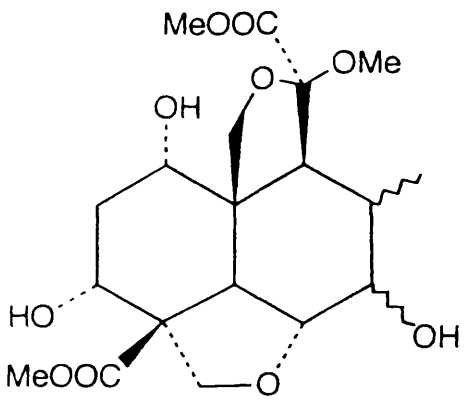
1 R=Ac ; R^1=R^2=H
2 R=Ac ; R^1=R^2=T ; 22,23-dihydro-
3 R=Ac ; R^1=R^2=H ; 22,23-dihydro-
4 R=R^1=R^2=H
5 R=Ac ; R^1=H ; R^2=OEt ; 22,23-dihydro-
6 R=Ac ; R^1=Br ; R^2=OEt ; 22,23-dihydro-



7



9



8

Fig. 3.7. Clearance of $^{3}H_2$-dihydroazadirachtin from the locust haemolymph in the presence of synthetic partial structural analogues.

The tracer dose of tritiated dihydroazadirachtin (**2**) was injected in the presence of 1000 fold concentrations of the furanoid ring moiety (**9**), and 1000 fold concentration of decalin ring moiety (**8**). The results were almost identical in both cases, and so only the results for the decalin fragment are shown. The results are the mean (± SD) of 5 individual locusts. The results for the tracer alone are included for comparison(---).

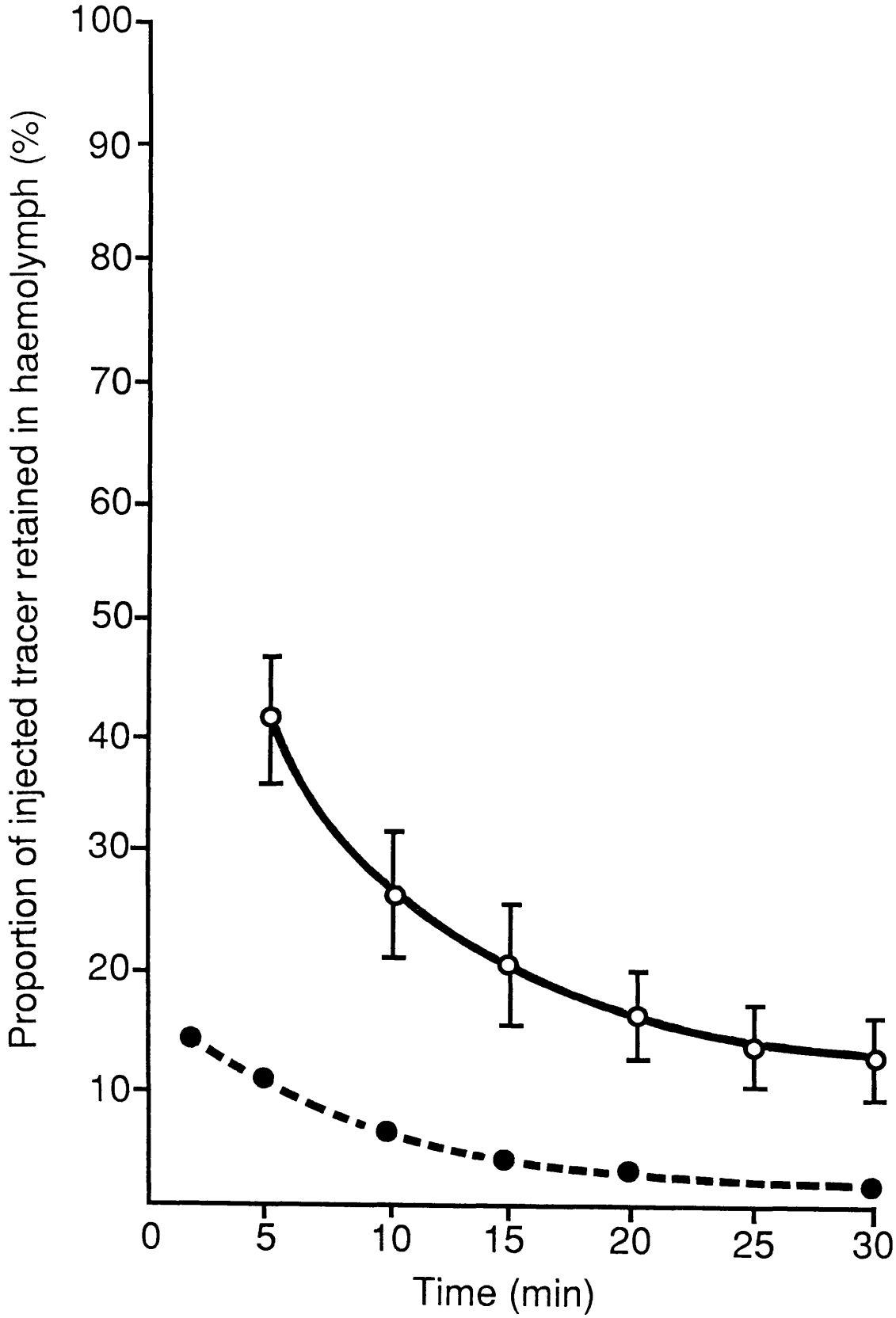

Proportion of injected tracer retained in haemolymph (%)
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
Time (min)

Fig. 3. 8. Semilogarithmic representations of the characteristics of the uptake of $^{3}H_2$-dihydroazadirachtin from the locust haemolymph in the presence of possible inhibitors.

The ordinate represents the $\log_{10}$ of the percentage of the total injected dose of tritiated tracer dihydroazadirachtin remaining in the haemolymph at various times. The results are derived from Figs .3.4, 3.5, and 3.7. The scale up to 2min has been enlarged in order to clarify the purely theoretical rapid initial uptake, based on instantaneous complete mixing of the tracer in the haemocoel, indicated as broken lines.

Figures above the lines indicate the relevant rate constant in arbitrary units. min^{-1}, and are included only for purposes of comparison.. The analogues are 4000 fold concentration of dihydroazadirachtin (●), 4000 fold concentration of azadirachtin (□), 1000 fold concentration of 3- deacetylazadirachtin (■), 1000 fold concentration of dihydroazadirachtin (○), 1000 fold concentration of furanoid ring (▽), 1000 fold concentration of 3- deacetylsalannin (▲). The results for tracer alone (△) are included for comparison.

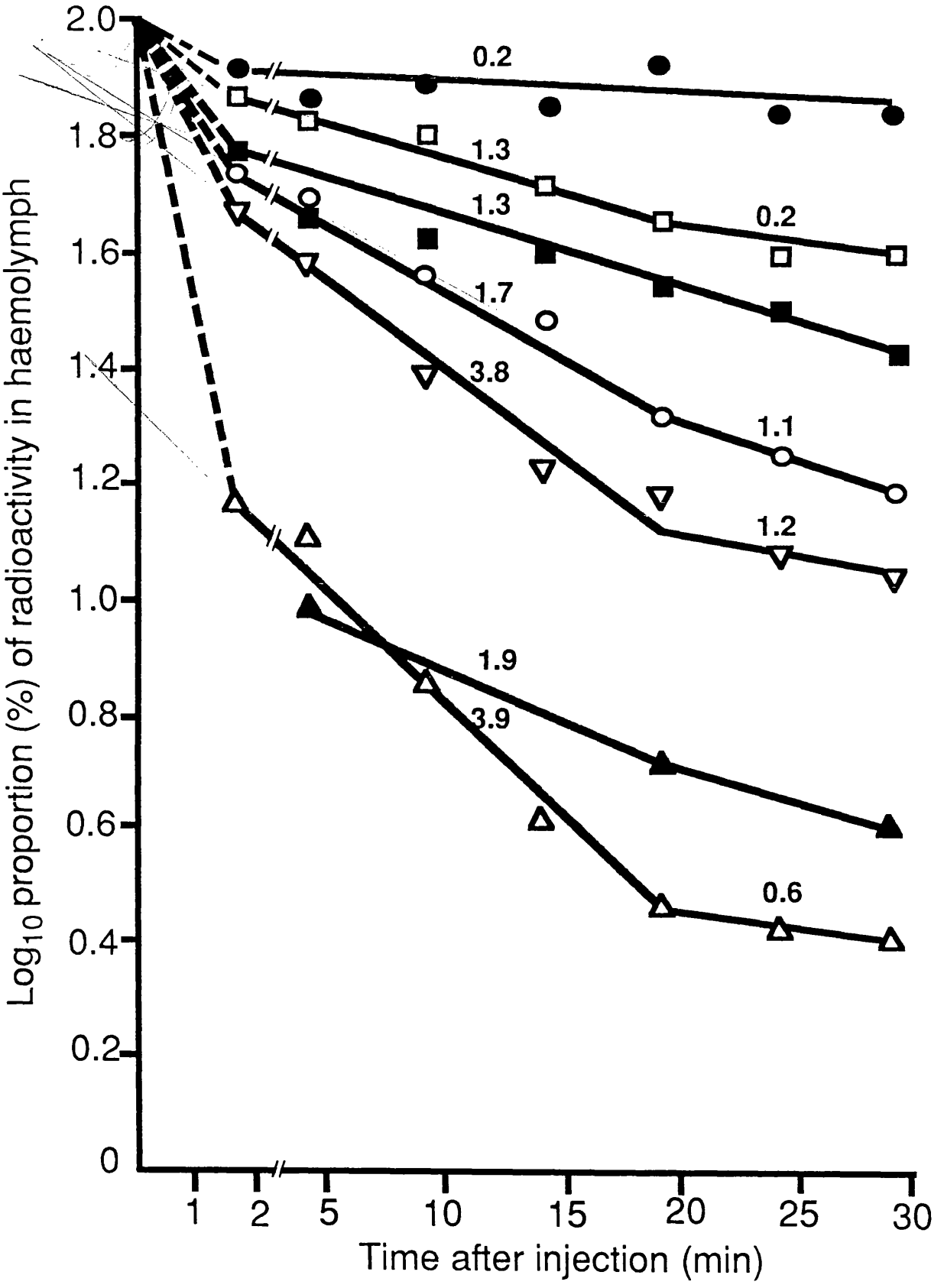

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Log10 proportion (%) of radioactivity in haemolymph
0.2
1.3
1.3
0.2
1.7
3.8
1.1
1.2
1.9
3.9
0.6
1
2
5
10
15
20
25
30
Time after injection (min)

Fig. 3. 9 Metabolism of $^{3}H_2$-dihydroazadirachtin by the locust *S. gregaria* .

The figure represents a silica TLC of radio-labelled compounds extracted from the locust 1 day after injection of tritiated dihydro compound.The chromatogram was run in 100% ethyl acetate in the direction shown and the radio activity located by the radoiochromatoscanner. The results are only semiquantitative.

Radioactivity (cpm)
Origin
Front
3H dihydroazadirachtin

Table 3.3 Metabolism of injected [22,23-$^3H_2$]-dihydroazadirachtin in the locust tissues

Tissue	% of unchanged dihydroazadirachtin	% of unidentified metabolite
Crop	45.1±6.5	54.9±5.7
Hepatic caeca	77.7±8.1	22.3±3.2
Malpighian tubules	79.1±7.3	20.9
Hind gut	88.2±6.9	11.8±0.9
Rest of the body	54.8±4.4	45.2±2.5

Notes: A tracer dose of tritiated dihydroazadirachtin (0.6nmol) was injected into the haemocoel of the adult locusts. After 7 days, the tissues were dissected and homogenised in 90% methanol to extract labelled material. Thin layer chromatography was carried out, each tissue using 100% ethyl acetate as a solvent.

3. 4. DISCUSSION.

The most significant of the results presented here are those which show the speed and apparent specificity of uptake of a low concentration of radiolabelled dihydroazadirachtin from the haemolymph into the tissues of the locust. If mixing were instantaneous, the concentration of the tracer would not have exceeded $1.2x10^{-6}M$ in the body cavity. In fact, it required 5 minutes for the injected tritiated inulin to be evenly distributed throughout the haemolymph, by which time almost 90% of the injected derivative of azadirachtin had been removed from the haemolymph. Although this confirms the previous qualitative observations of Rembold et al., (1983) with the locust , this phenomenon is not universal, as the concentration of the tritiated dihydro derivative in the blood of *Rhodnius* remains high for several days (Garcia et al, 1989).

Some part of this uptake must be by non-specific diffusion and binding, but the theoretical partition coefficient between octanol and water for dihydroazadirachtin, (logP = -2.1; D.E. Leahy, personal communication), suggests that it is not at all lipophilic. The effect of low temperature showed that most of the uptake was not due to diffusion, but suggested a carrier-mediated process. The effect of high concentrations of unlabelled dihydroazadirachtin and other structural analogues confirmed the impression of the specificity of the uptake mechanism. About 80% of the uptake of the tracer was inhibited by a concentration of unlabelled compound four thousand times that of the tracer dose. This suggests that the carrier mechanism is both specific and saturable, and that only 20% of the total uptake is likely to be non-specific.

The uptake of the tracer is clearly exponential, but does not follow a simple pattern. There may be distinguished at least two and possibly three separate exponentials with different rate constants. The most elusive is the uptake at times of less than 2 minutes, some of which must be non-specific, although not all, as indicated by the inhibitory effects of analogues. In view of the fact that the label is taken into many tissues in the locust this complexity is unsurprising. The results indicate that whatever the nature of the various mechanisms, all are likely to be specific although their pharmacology may be slightly different..

Structural analogues in large excess inhibit the uptake of the tracer to different extents. The semilogarithmic plots can be interpreted as indicating that the analogues may have different effects on different uptake mechanisms. Table 3.4 shows that there is a remarkable similarity between the capacity of analogues to inhibit the development of *Epilachna* larvae (Jacobson,1988), and to inhibit uptake of injected dihydroazadirachtin into the locust tissues. It would be unwise to lay too much emphasis on this similarity, as the time chosen (20 min after injection) is arbitrary, and the parallel is less true at shorter times, but neverthless it

suggests that there may be specific receptors for azadirachtin drivatives, which transcend species barriers. One certain finding is that salannin, which has no effect on *Epilachna* development (Jacobson, 1988) does not compete at all for uptake, in the locust, and it may be speculated that this may be associated with its lack of effect. Another interesting observation is that azadirachtin, the parent compound, inhibits uptake much less effectively than the same concentration of dihydro-azadirachtin. This too, is reflected in the biological assay results with *Epilachna* (Rembold, 1988). Whatever else this might imply, while the two compounds may have the same general biological effect, there are differences in their quantitative pharmacology, which will have to be taken into account.

Table 3.4. Comparison of structural analogues of azadirachtin in a bioassay and as competitors of tissue uptake of $^{3}H_2$-dihydroazadirachtin.

Compound	Biological effect*	Uptake**
3-Deacetyazadirachtin (**4**)	100	100
23-Ethoxy-22,23-dihydroazadirachtin (**5**)	80	90
22,23-Dihydroazadirachtin (**3**)	32	54
3-Deacetylsalannin (**7**)	<4	6

* The biological effect is based on the reported values for the concentrations preventing metamorphosis in *Epilachna* (MC50 values) (Rembold, 1989). Reciprocals were taken of the values, and they are expressed as a % of the most potent compound.

** These values represent the relative effectiveness of the derivatives in preventing clearance of a tracer dose of $^{3}H_2$-dihydroazadirachtin from haemolymph of *Schistocerca*. Each was present in 10^3 excess concentration over the tracer, and the figures are based on the label in the haemolymph 20 min after injection. Inhibition is expressed as a % of the most effective compound.

Comparison of the partial analogues(Fig.3.6, **1-6**), the decalin and furanoid ring systems, showed that they were equally effective , and similar to dihydro-azadirachtin itself, in inhibiting the uptake of the tracer. This suggests that uptake into the locust tissues depends on receptors which have rather similar affinities for both major parts of the whole molecule. This is interesting in light of the suggestions that the different parts of the molecule have distinct biological effects (Aldous, 1992).

The survey of uptake into different tissues gives a broader picture than previous work, and suggests that although the previous results showing uptake into the alimentary canal and nervous tissues are confirmed, there is also large uptake into the carcase, probably into the fat body. Rembold (1988) found that the highest level of label was found in the Malpighian tubules, (73% of the total after 5 days) but in the results reported here the activity in this tissue was exceeded by others. The fact that only careful washing of the caeca after incisions had been made in them, reduced the extent of labelling, is consistent with the compound being first cleared by the Malpighian tubules, and then carried forward into the caeca (Dow, 1981) where it is retained over several days. Although there are differences in detail, the results broadly agree with those of Rembold (1983,1988,1989).

The evidence suggests that the locust is not very effective at metabolising or excreting the azadirachtin . As was observed by Rembold, almost all excretion of label took place within 24 hours of injection. We could find no evidence that the labelled material was actively concentrated in the primary urine. The material which had been taken into the tissues remained quite constant over a period of seven days. It was displaced to only a very small extent by a large excess of unlabelled dihydroazadirachtin, although the full extent of displacement may be masked by the

toxic effects of the compound which prevented further excretion. Metabolism too was limited, mainly to fat body, and, to a lesser extent, crop. In many tissues the label extracted after seven days was almost unmetabolised. Although there was evidence that the label was present in a polar metabolite, indicating that dihydroazadirachtin can be metabolised by the locust, no identification of this material is currently possible. Preliminary results, however, suggest that it is produced by a cytochrome P450 monoxygenase (Wargent, E. personal communication), which would be consistent with high metabolism in the fat body, and with the polar nature of the product.

At the moment it is impossible to be precise about the nature of the uptake mechanism, or how it relates to individual tissues. It is even less clear how it might finally relate to biological effects. The competition studies do, however, confirm the contention which underlay the aims of the work, namely, that there are receptors in the locust which show a high specificity for azadirachtin and its analogues.

CHAPTER 4

EFFECT OF AZADIRACHTIN AND DIHYDROAZADIRACHTIN ON THE FUNCTION OF THE MALPIGHIAN TUBULES OF THE LOCUST, *S. GREGARIA*.

4.1. INTRODUCTION

The toxicology of azadirachtin is not well understood, but the previous biological work has implied an action of azadirachtin at the neuroendocrine level. As described in Chapter 3, it is clear that (22,23)-dihydroazadirachtin induces the same biological effect as azadirachtin (Rembold, et al. 1988), and the present study also showed that the Malpighian tubules contained a higher concentration of azadirachtin than that in any other tissue of the alimentary canal. In the Malpighian tubules the dihydroazadirachtin extracted after 7 days injection was almost unmetabolised. Malpighian tubules play an important role in the excretion and clearance of drugs, toxins and other foreign compounds, and also maintain the haemolymph chemical composition and water balance. Autoradiographic studies of Malpighian tubules provide evidence for the accumulation of ^{3}H-dihydroazadirachtin in the basal membrane and the cytoplasm around the nucleus. The Malpighian tubules of the locusts have a uniform histological structure over the length of the tubule, and it is similar to that of many other insect species. The basal region where dihydroazadirachtin accumulates, includes the basal cell membrane and its infoldings into the cell. During the process of fluid secretion this compound would pass through the tubules and enter the lumen for excretion (Rembold et al, 1988).

Hitherto, however, there has been no physiological effect of azadirachtin and its derivatives detected on the Malpighian tubules.

4.1.1. Malpighian tubules : water balance and hormones

Insects, in common with many other invertebrates, are much more tolerant of changes in the constituents of their fluid components than are vertebrates. Nevertheless, solute concentrations must be controlled. The Malpighian tubules form a primary urine, and are in many ways analogues of the glomerulus of the

mammalian kidney. They produce, both by passive secretion and specific active transport systems, a continuous flow of primary urine which contains all the small molecular weight materials present in haemolymph. The primary urine enters the gut where some components may move forward for reabsoption in the mid gut (Dow, 1981). The rest of the fluid mixes with the gut contents and moves through the hind gut to the rectum, where selective reabsorption of water, ions and metabolites occurs by active transport mechanisms (Phillips, 1980, 1981). It is in the rectum that the excreta finally becomes hyper- or hypo-osmotic to the haemolymph, due to changes in the relative rates of water and ion absorption. The formation of urine in insects has been investigated extensively in isolated Malpighian tubules, using an *in vitro* technique (Maddrell et. al., 1973). This allows measurements to be made of effects of the composition of the bathing medium upon the composition and rate of formation of primary urine.

To promote excretion, insects release at least one diuretic hormone, ie. a neurohormone which is synthesized by cerebral neurosecretory cells and released from the storage regions of the corpus cardiacum (Goldworthy, 1969). The diuretic hormone acts upon the Malpighian tubules to increase the volume of urine secreted, without altering its composition.

When the locust feeds, the diuretic hormone is released into the haemolymph and acts upon the excretory system. Thus at times of water intake and when the neurosecretory system responds to feeding stimuli, the excretory system is made to function so as to promote water loss from the insect. When the insect stops feeding, neurosecretion is no longer released from the corpora cardiaca, and the rate of excretion falls and more water is absorbed through rectal wall..

It has been shown that cyclic AMP acts as an intracellular second messenger in the Malpighian tubules of insects, and mediates their response to some diuretic peptides (Maddrell, et. al., 1971). Tissue extracts containing diuretic

peptides increase the intracellular concentration of cAMP in isolated tubules, and stimulate adenyl cyclase activity in Malpighian tubule plasma membrane preparations (Morgan, et. al., 1985). Solute pumps are likely to be activated by protein kinases stimulated by increased concentrations of cAMP. This can be mimicked by addition of cAMP to the bathing medium, which markedly increases the rate of secretory activity of the tubules of locust *in vitro* (Mordue, 1980). An advantage of Malpighian tubules *in vitro* is that they are sensitive to externally applied cAMP, unlike most mammalian tissue preparations.

Assays based upon the measurement of changes in cAMP production by isolated tubules or of adenyl cyclase activity in Malpighian tubules plasma membrane preparations, can be used to screen chromatography fractions for potential diuretic peptides (Rafaeli, et. al. 1984). The use of such assays presupposes that cAMP acts as an intracellular second mesenger, but this may not be true for all diuretic peptides. For example, although in *Locusta migratoria* the diuretic peptides stimulate cAMP production in isolated Malpighian tubules, the diuretic hormone of *Manduca sexta* appears to work independently of cAMP (Kataoka, et. al., 1989). Similarly, of the two diuretic hormones described in the corpora cardiaca of locust, only diuretic peptide I stimulated adenyl cyclase activity and elevated intracellular levels of cAMP. Diuretic peptide II worked via a calcium-dependent mechanism (Morgan, et. al., 1985).

The phosphodiester bond in cAMP is hydrolysed by a specific phosphodiesterase enzyme to form AMP, which does not activate the protein kinase. These cyclic nucleotide phosphodiesterases are inhibited by methylxanthines such as theophylline and caffeine. These compounds prolong the responses to cAMP by slowing the degradation of this intracellular messenger.

4.1.2. Active transport of acidic dyes by insect Malpighian tubules.

Malpighian tubules are freely permeable to organic solutes of low molecular weight, and in some insects to molecules as large as inulin. Organic solutes may be passively or actively secreted into the tubule lumen, providing a mechanism for the removal of unwanted material from the haemolymph (Maddrell, et al. 1974).

Insect Malpighian tubules have long been known to concentrate acidic dyes such as amaranth and indigo carmine. Dye secretion by Malpighian tubules has been used by several workers as an indication of the state of this activity. It has been recorded that insect Malpighian tubules actively excrete amaranth when it was administered by injecting into the haemolymph of the locust (Maddrell, et al., 1974) and this mechanism is specific, saturable, and obeys Michaelis-Menton kinetics.

Comparision of the rate of excretion of amaranth following neurosecretory cell cautery and allatectomy suggests that the neurosecretory system exerts a direct influence upon excretion. The rate of excretion of amaranth through the Malpighian tubules of *S. gregaria* is increased by extracts of corpora cardiaca (Mordue, 1968). The rate of excretion varies with the developmental state of the locust, and this is a result of variations in neurosecretory activity with various stages of development. For example, to maintain the high rate of excretion in maturing females, diuretic hormone is released at a much faster rate than in young males where the level of excretion is much lower.

4.1.3. The aims of the experiments

It has been recorded that Malpighian tubules retained a high concentration of azadirachtin, and on this basis, it might perhaps be anticipated that there is some action of azadirachtin upon the excretory system. Therefore the preliminary experiments were designed to investigate whether or not azadirachtin has an effect

upon primary urine secretion or on dye transport by the Malpighian tubules of *S. gregaria*. This may provide

(i) a simple, fast, *in vitro* screening method to assess biological differences of azadirachtin and its derivatives,

(ii) a clue to the sub-cellular action of azadirachtin

4.2. METHODS AND MATERIALS

4.2.1. Insects

Insects were maintained under laboratory conditions as described in Chapter 3 and female locusts were used for all the experiments, because the rate of excretion is faster in female locusts than in male locusts.

4.2.2. Insect saline

Saline containing KCl, 10mM; NaCl, 140mM; $CaCl_2$, 2mM; glucose, 10mM; sucrose, 100mM; to bring the osmotic pressure of solution to value of the locust haemolymph, and MOPS buffer, 100mM; (buffered to pH6.9) was used in this experiment as a locust physiological medium. Theophylline (10^{-3}M) was also used throughout the experiment to prevent hydrolysis of c-AMP.

4.2.3. Stimulants

Solution of 10^{-1}M cAMP (adjusted to pH=6.9) was added to the saline to give a final concentration of 10^{-3}M in this experiment, to maximally stimulate water secretion.

4.2.4. *In vitro* preparation of Malpighian tubules

The method used to follow secretion of Malpighian tubules was an *in vitro* modification of method used by Mordue et al. (1969).

Before each experiment the locusts were starved for twelve hours to empty the gut and eliminate acute diuretic effects. They were anaesthetised under CO_2, and the gut was dissected out, placed in locust saline and carefully cleaned under the microscope by removing fat bodies. The gut was ligatured immediately anterior to the point of the entry of the tubules. A graduated micropipette (5µl), pretreated with the antiwetting agent 2% dimethyl dichlorosilane, to prevent surface tension of water, was then inserted into the gut, immediately posterior to the Malpighian tubules, with the help of forceps, and ligatured in place. The aim was to isolate as small a volume of gut as possible, so as to ensure that any secreted water was forced into the micro-pipette without accumulating in the gut. The ligatured region of hind gut was excised and immersed in 2ml of locust saline. The saline was maintained at a constant temperature of 30°C, and the rate of Malpighian tubules water secretion was estimated by following the movement of water in the micro-pipettes. This was expressed as $\mu l.h^{-1}$. The experiment was continued for 4 hours.

The following substances were added to the medium separately and differences of rates of water secretion were measured.

(i) 10^{-3}M cAMP

(ii) 10^{-3}M azadirachtin

(iii) 10^{-3}M dihydroazadirachtin

4.2.5. Effect of azadirachtin on uptake of amaranth by Malpighian tubules.

Experiments were carried out both *in vivo* and *in vitro*.

4.2.5.1. *In vivo* experiments

4.2.5.1.1. Application of azadirachtin and amaranth

The effect of azadirachtin varies with the time after injection of azadirachtin. Therefore two basic experiments were carried out: an acute experiment; in which the effect was examined 5 min after injection of azadirachtin and a chronic experiment; in which the effect was examined 12 hours after injection of azadirachtin.

a) Injection of azadirachtin

0.33×10^{-6}M and 0.2×10^{-3}M concentrations of azadirachtin were initially dissolved in ethanol separately, then they were diluted with distilled water to 10% v/v and 70% v/v ethanol/water respectively (70% ethanol was used to make the high concentration of azadirachtin, because the higher concentration of azadirachtin failed to dissolve in 10% ethanol). 3µl of each solution contained 1nmol and 1000nmol of azadirachtin respectively and this amount was used for the injection of each locust. Control locusts received equal amount and equal percentage of ethanol/ water (It was found that high concentrations of ethanol reduce the rate of clearance of amaranth from the haemolymph).

(i) Acute experiment

The different concentrations of azadirachtin were injected into each locust 5min before injection of amaranth. Injections were made through the abdominal intersegmental membrane using a Hamilton 10µl syringe. The amounts of azadirachtin used were

(1) 1 µg/locust

(2) 100 µg/locust

(ii) Chronic experiment

The same amounts of azadirachtin as in the acute experiment were injected 12 hours before injection of amaranth

The volume of the haemolymph in the each locust was assumed to be 500µl (See Chapter 3). This figure was used to determined the theoretical initial concentrations (assuming instantaneous complete mixing) of azadirachtin in the haemolymph. They were as follows:

(a) $2x10^{-9}M$ (b) $2x10^{-6}M$

(b) Injection of amaranth

A 50mM solution of amaranth was prepared by dissolving 302mg in 10ml of insect saline. 20µl of 50mM amaranth was injected using Hamilton 25µl syringe and 3µl samples of haemolymph were taken from the hind coxa of the locust using a 5µl micropipette every 10min. Both experimental and control locusts received the same amount of amaranth and 5 locusts were used for each experiment. Previous experiment showed that the rate of clearance of amaranth in the medium is maximum at concentration > $1.5x10^{-3}M$. Therefore, when the theoretical concentration of amaranth in the haemolymph was calculated, this figure was above that for saturation ($2x10^{-3}M$).

4.2.5.2. *In vitro* experiments

When the higher concentration of azadirachtin (100 µg) was used, the insect rapidly became morbid, and this may have reduced the rate of haemolymph circulation and mixing. Therefore, in order to eliminate gross physiological effects, an *in vitro* experiment was carried out.

4.2.5.2.1. Effect of azadirachtin on uptake of amaranth by Malpighian tubules .

A sample of azadirachtin (10 μg) was injected into each locust 12 hours before injection of amaranth. Then the locusts were anaesthetised under CO_2 and the gut was dissected out, placed in locust saline and carefully cleaned under the microscope. The gut was ligatured anterior to the point of entry of the tubules and posterior to the Malpighian tubules. Two preparations were used for each assay.

A sample of amaranth (2ml of 0.1mM), dissolved in insect saline, was maintained at constant temperature of 30°C and aerated throughout the experiment. The Malpighian tubules preparations were placed in the amaranth and absorbance was measured at every 10 min. Before measuring the absorbance, the medium was centrifuged for 1 min to make a clear solution. The concentration of the dye was measured spectrophotometrically at 550nm.

Control insects received injections of equal amount of 10% ethanol/water 12 hours before the start of the *in vitro* experiment.

4.2.5.3. Estimation of amaranth in the haemolymph by spectrophotometry

Samples of haemolymph (3μl) were taken from the base of hind coxa of the locust using 5μl micropipettes, and diluted into 1ml of water. The concentration of the dye was measured with a spectrophotometer at 550nm which is the λ_{max} of amaranth. Then the molar extinction coefficient (ε_{550}) of amaranth was calculated as $14.5 \times 10^2 l.mol^{-1}cm^{-1}$.

4.3. RESULTS

4.3.1. Effect of azadirachtin on secretion of primary urine

First, the unstimulated rate of Malpighian tubules water secretion was examined in insect saline, giving an average of basal rate of $1.5\pm 0.25\mu l/h^{-1}$ $(n=4)$. Although the addition of $10^{-3}M$ c-AMP increased the rate to 2.57 ± 0.78 $\mu l/h^{-1}$, the results were not significant ($p>0.05$).

The effect of dihydroazadirachtin and azadirachtin upon the rate of fluid secretion were compared in the absence and presence of c-AMP. On the basis of the results obtained neither dihydroazadirachtin or azadirachtin had any additive effect upon the rate of fluid secretion in the presence of cAMP (Table 4.1). Azadirachtin and dihydroazadirachtin added to the medium in the absence of c-AMP, did increase the rates of fluid secretion of Malpighian tubules to $2.75\mu l\pm0.30$. Both azadirachtin and dihydroazadirachtin showed similar effects. But again the high variability meant the stimulatory effect was not statistically significant.

4.4.2. Effect of azadirachtin on clearance of amaranth from the haemolymph.

Using amaranth dye as a marker solute, clearance from the haemolymph of *S. gregaria* was studied. Amaranth was rapidly excreted by the locusts, and it was completely eliminated from the haemolymph in 4-6 hours. Figs.4.1 and 4.2 show the effect of different concentrations of azadirachtin on the clearance of amaranth from the haemolymph 5min after injection of azadirachtin. The results show that both experiments with low and high concentrations of azadirachtin were similar and not statistically different from the controls.

Table-4.1 Effect of azadirachtin on water pumping through Malpighian tubules of *S. gregaria.*

Additions	Rate of fluid Secretion($\mu l h^{1}$)
Control	1.50±0.25
cAMP	2.57±0.50
dihydroazadirachtin	2.75±0.90
azadirachtin	2.50±0.85
cAMP 10^{-3}M + dihydroazadirachtin	2.75±0.58
cAMP 10^{-3}M + azadirachtin	2.45±0.60

Each result represents the mean value (±SD) of 5 individual locusts.The control consisted only insect saline. Final concentrations of c-AMP, azadirachtin and dihydroazadirachtin were 10^{-3}M. After 60min the movement of water in the micropipette was measured.

As the acute experiment showed no effect upon uptake of amaranth from the haemolymph, a chronic experiment was carried out for further investigation. Azadirachtin was injected into each locust 12 hours before injection of amaranth. The locust treated with a low concentration of azadirachtin (1nmol/locust) showed the same results as the control. No conclusion could be drawn for the higher concentration of azadirachtin due to the morbidity of the insects. The problem of mixing and circulation of haemolymph was overcome by doing the experiment *in vitro*. It has been shown that all the amaranth was taken up by the Malpighian tubules within 40mins. Fig.4.3 presents a semilogarithmic representation of the clearance of amaranth from the bathing medium via the Malpighian tubules of the locust.

Fig.4.1 Semilogarithmic representation of the effect of 10^{-4}M azadirachtin on the clearance of amaranth from the haemolymph of the locust.

50mM amaranth (20µl) was injected into the haemocoel of locusts, and 3µl samples removed for estimation of the concentration of the dye at 550nm at various times. The concentration at 5min was taken as 100%.

5 minutes before injection of the dye 3 µl volumes of 0.33mM azadirachtin in 10% ethanol/water were injected, giving an estimated maximum concentration of azadirachtin in the haemolymph of 10^{-4}mM (●).

The control locust (○) received only 3µl of 10% ethanol/water.Each point is average (±SD) of 3 locusts.

The ordinate represents the $\log_{10}$ of the percentage of the amaranth in the haemolymph at various times.

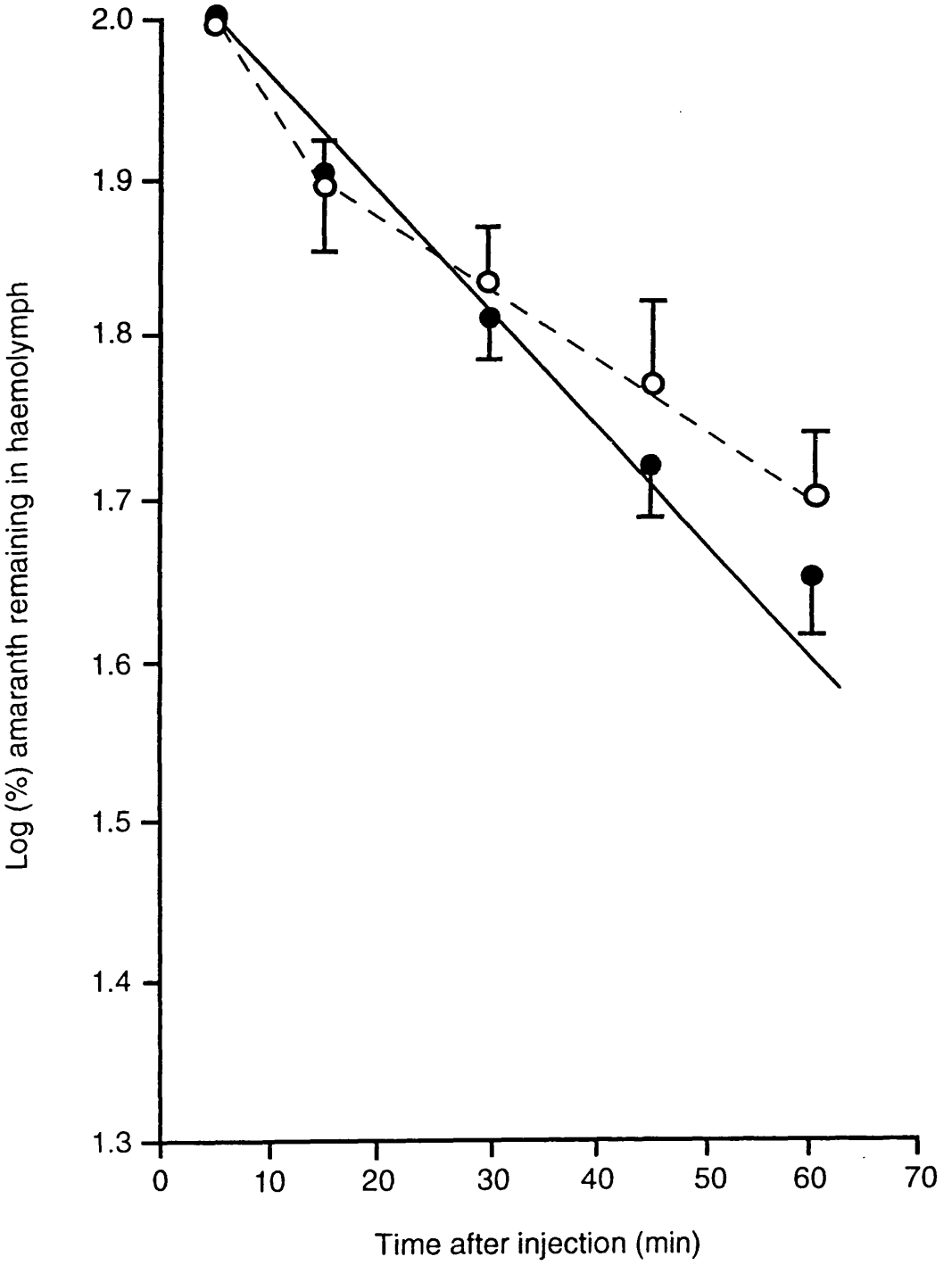

2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
0
10
20
30
40
50
60
70
Log (%) amaranth remaining in haemolymph
Time after injection (min)

Fig.4.2 Semilogarithmic representation of the effect of $2x10^{-3}M$ azadirachtin on the clearance of amaranth from the haemolymph of the locust.

50mM amaranth (20µl) was injected into the haemocoel of locusts, and 3µl samples removed for estimation of the concentration of the dye at 550nm at various times. The concentration at 5min was taken as 100%.

5 minutes before injection of the dye, 5 µl volumes of 200mM azadirachtin in 70% ethanol/water were injected, giving an estimated maximum concentration of azadirachtin in the haemolymph of 2mM (●).

The control locust (○) received only 5µl of 70% ethanol/water.Each point is average (±SD) of 3 locusts.

The ordinate represents the $\log_{10}$ of the percentage of the amaranth in the haemolymph at various times.

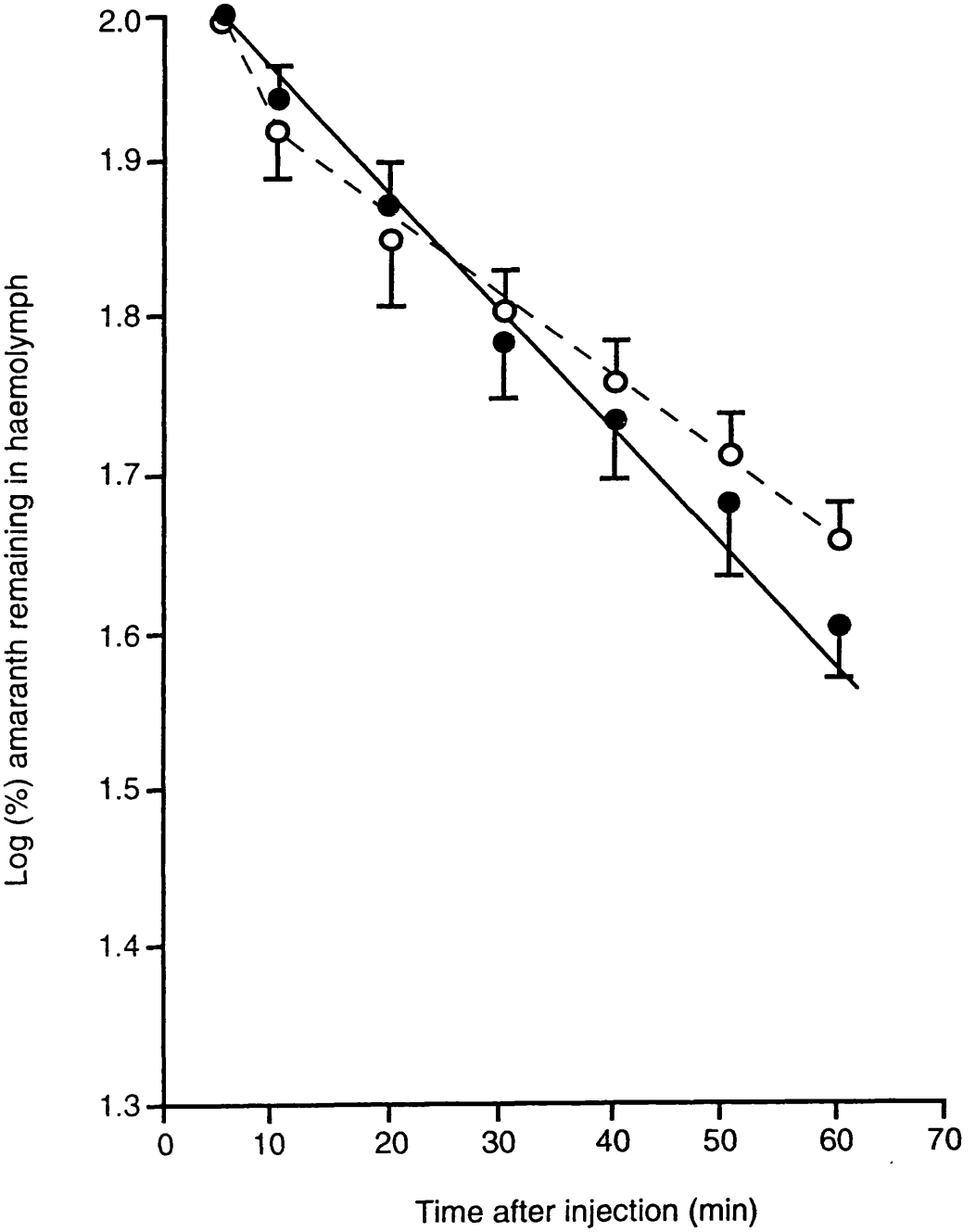

2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
0
10
20
30
40
50
60
70
Log (%) amaranth remaining in haemolymph
Time after injection (min)

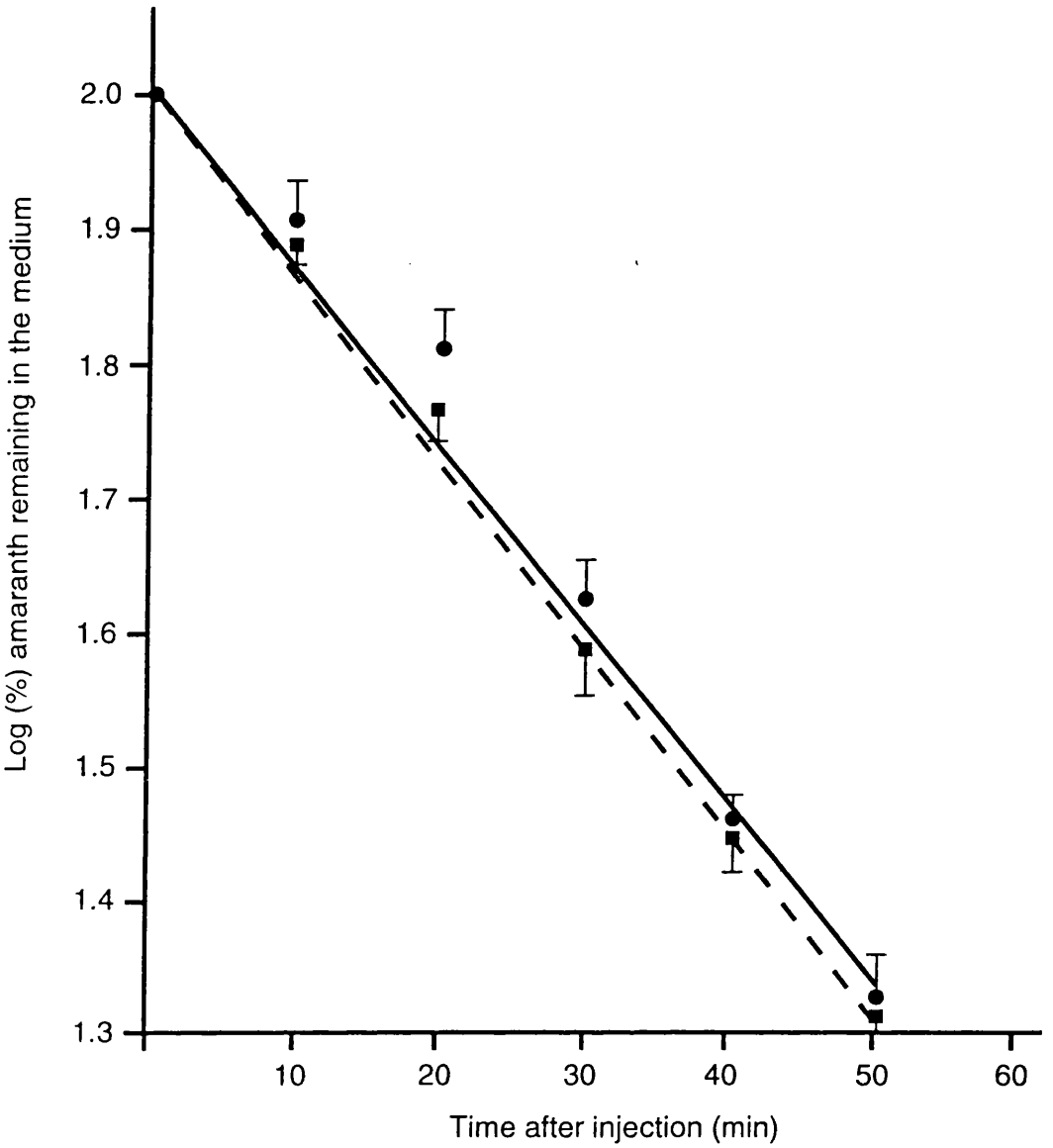
Fig.4.3 Semilogarithmic plot of effect of azadirachtin on the rate of the fluid secretion by locust Malpighian tubules *in vitro*.

Each experiment contained two Malpighian tubules preparation in 2 ml of medium with 0.1mM amaranth. The concentration of the dye was measured at 550nm at various times. The concentration at zero time is 100%.

12 hours before application of the dye 5µl volumes of 20mM concentration azadirachtin (●) in 10% ethanol/water were injected.

The control locust (■) received only 5µl of 10% ethanol/water. Each point is average of (±SD) of 3 determinations.

The ordinate represents the $\log_{10}$ of the percentage of the amaranth in the bathing medium at various times.


2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
Log (%) amaranth remaining in the medium
10
20
30
40
50
60
Time after injection (min)

The slope of the line gives a measure of the rate of uptake of the dye and the intercept gives the theoretical initial concentration. The results show that azadirachtin added to the medium has no significant effect upon clearance of amaranth from the medium.

4.5. DISCUSSION

4.5.1. Effect of azadirachtin on secretion of primary urine

Diuretic factors act upon Malpighian tubules to increase the volume of fluid secreted. This effect is mimicked by changing the concentration of cAMP in the bathing medium. The rate at which Malpighian tubules of locusts secreted fluid is maximal when the bathing solution contains a concentration of cAMP greater than 10^{-4}M (Morgan et al., 1985).

Although previous and present results indicated high amounts of azadirachtin binding to the Malpighian tubules, neither azadirachtin nor dihydroazadirachtin had any additive effect upon the rate of secretion of primary urine through the Malpighian tubules in the presence of stimulant.

In the absence of c-AMP both terpenoids increased water excretion. Both azadirachtin and dihydroazadirachtin seem to have an acceleratory effect upon the rate of water secretion. It was difficult however, to draw firm conclusions, because the results were very variable.

4.5.2. Effect of azadirachtin on uptake of dye

The present results indicate that the uptake of amaranth is exponential by a single mechanism with one rate constant and the experiment in which the tubules are labelled with amaranth clearly suggests that the Malpighian tubules are the only tissue responsible. Excretion of amaranth is increased by extracts containing

neurohormones. It is very likely that these hormones controlling excretion through the Malpighian tubules are neurosecretory in origin since extracts from the storage lobe regions of the corpora cardiaca are very potent in increasing excretion. To maintain high rates of excretion in maturing females, diuretic hormones are being released at a much faster rate than in young males where the level of excretion is much lower (Mordue, 1968).

The control experiment of the *in vitro* preparation shows that amaranth is rapidly secreted by the Malpighian tubules of the female locusts. The degree to which amaranth is concentrated depends critically on the passive permiability of the tubule wall. For example unstimulated *Rhodnius* Malpighian tubules concentrated amaranth in the lumen at a level which was up to 300 times higher than in the bathing fluid (Maddrell, et al., 1974).

According to the previous work more than 14% of the injected dihydroazadirachtin is removed from the haemolymph 2 min after injection and a certain amount of it binds to the Malpighian tubules. Although uptake of azadirachtin is very fast, the acute experiment shows there is no immediate inhibitory effect on clearance of amaranth through the Malpighian tubules even at high concentration.

This experiment failed to demonstrate any interference of azadirachtin on clearance of amaranth from the bathing medium since the results were similar to control experiment. Therefore further experiments are required to study the physiological effect of azadirachtin on Malpighian tubules of the locust.

CHAPTER 5

EFFECT OF AZADIRACHTIN ON INCORPORATION OF RADIOLABELLED AMINO ACIDS INTO THE PROTEINS OF THE LOCUST

5.1. INTRODUCTION

Previous research work on the mode of action of azadirachtin, reviewed in the Introduction, has clearly indicated that one major difficulty is the diversity of effects, some of which differ from one insect species to another. Some of these effects are dramatic, e.g. the inhibition of various stages of development of holometabolous insects, but some are much more subtle, and cause only rather ill-defined reductions of "fitness".

The studies on uptake of the radio-labelled tracer reported in Chapter 3 have further indicated that the compound is taken up, apparently with great specificity, into nearly all tissues examined. This further increases the difficulty of isolating common actions. Some tissues, however, were shown to be more highly labelled than others e.g. Malpighian tubules, corpus cardiacum, brain, fat body etc. Although this might be considered to be a useful guide to possible physiological effects, these may still prove elusive. For instance, it has been reported both here and by others that the Malpighian tubules show high levels of binding. This does not appear to be merely a process of excretion, as the binding seems long-term, but it has been impossible to show any effect of the azadirachtin on other excretory processes.

The accumulation in the brain and corpus cardiacum however, may give a clue to one of the major sites of action, as noted by others (Rembold et al., 1989). Many of the effects of azadirachtin on various aspects of development are on processes ultimately controlled by neurohormones. There is ample evidence that most of these are peptide in nature (Giradie, et al., 1987a). Previous work has already shown that azadirachtin has an effect on protein metabolism in the brain and corpus cardiacum (Subramanyam et al., 1989b).

There is evidence from others that azadirachtin may interfere with protein metabolism in other tissues, e.g. inhibition of secretion of trypsin in the midgut of

Manduca sexta caterpillars (Timmins et al., 1992), immune-depression in the haemolymph of *Rhodnius prolixus* (Azambuja et al., 1991). There is no doubt that protein metabolism is important to all aspects of development, whether through the formation of neurohormones or in the developing of the mature ovaries in the female. It may be that interference with protein and peptide biosynthesis could represent a common mechanism of azadirachtin action in many insect tissues.

5.1.1. Effect of azadirachtin on incorporation of amino acids into the proteins of the locusts

Very little work has been done on inhibition of protein synthesis by azadirachtin. The neuroendocrine proteins from the corpus cardiacum have been characterised in order to elucidate the quantitative and qualitative differences in their biosynthesis due to azadirachtin treatment (Subrahmanyam et al., 1989b). Radio-labelled cysteine incorporation demonstrates that the azadirachtin-treated locusts have a very low turnover of neurosecretory protein in the corpus cardiacum. In the control locusts, the transport of labelled protein from the brain to the corpus cardiacum and its subsequent release are at a higher level (Subrahmanyam et al., 1989b).

The electrophoretic pattern of neurosecretory proteins of the locust was studied. Six protein fractions incorporating labelled cysteine has been detected in the extracts of the storage lobes of the corpus cardiacum of both control and azadirachtin-treated locusts, suggesting that azadirachtin treatment does not induce any qualitative difference in the synthesis of neurosecretory proteins.

5.1.2. Effect of azadirachtin on the gut and the haemolymph

The mid gut is concerned with the digestion and absorption of food. In insects, as in other animals, foodstuff is digested physically and then chemically degraded into sub-units to facilitate absorption into the blood stream. The enzymes responsible for the chemical phase of digestion occur in the salivary gland and mid-gut secretion as well as within the mid gut tissues. The carbohydrases, proteases and lipases present in insects are, in general, similar in properties to those in vertebrates.

Disfunction of the insect gut following azadirachtin treatment is now well established and recent work on *S. gregaria* and *L. migratoria* has demonstrated histologically that the midgut epithelial cells show signs of necrosis, seen as a rounding-up and swelling of the cells and organelles, with some vacuolization and cell bursting (Cottee, 1984; Nasiruddin et al., 1993). It is likely that such disrupted tissues would function abnormally and enzyme secretion and nutrient absorption would be disrupted (Mordue et al., 1993).

The principal gut proteinase in *Manduca sexta* is a typical trypsin-type endoprotease. The amount of trypsin activity in the midgut contents of azadirachtin-treated insects was 20% of that seen in controls. The decrease of trypsin activity is not due to direct inhibition of the enzyme by azadirachtin. It has been shown that azadirachtin prevents the secretion of the enzyme into the midgut lumen and release of enzyme that has already been synthesised may be inhibited (Timmins et al., 1992). In addition, azadirachtin reduces the activity of digestive enzymes such as protease, amylase, and invertase in both *Spodoptera litura* (Ayyangar et al., 1989) and *Euproctis fraterna* (Sridhar et al., 1989).

It has been recorded that azadirachtin influenced immune reactivity of the haemolymph protein in larvae of *R. prolixus* when *Enterobacter cloacae* B12 strain were inoculated into the insects. Azadirachtin affects the immune reactivity as

shown by the following: significant reduction in numbers of haemocytes after challenge with *E. cloacae*; the insects decreased ability to produce antibacterial and lysozyme activity in the haemolymph when inoculated with bacteria; the reduced ability of azadirachtin-treated insects to destroy the primary infection caused by inoculation with *E. cloacae*. In this case, an alternative explanation could be that azadirachtin decreased the immune defence system due to general inhibition of the insect metabolism, suggesting that the insect may have a diminished ability to undertake haemolymph protein synthesis (Azambuja et al., 1991).

5.1.3. Protein synthesis in the development of locust ovaries

5.1.3.1. Protein metabolism of insect fat body

The fat body performs many of the functions of the vertebrate liver, including amino acid metabolism, and making the residues available for protein synthesis by fat body and other tissues such as haemolymph and ovary. Studies of the incorporation of radiolabelled amino acids into proteins can provide very useful data on the pathways of biosynthesis and metabolism. The pattern of incorporation obtained for the various precursors show that the fat body is able to use a wide range of precursors for the formation of fat and protein, although, as would be expected, glycine and leucine were more readily incorporated into protein than the other precursors (Clements, 1959) because of the high concentration of these two amino acids in the haemolymph of the locusts.

In the normally developing adult female desert locust, just after the final ecdysis, the incorporation of glycine into the fat body protein is low for seven days, then the incorporation rate increases rapidly from 7 days to 14 days after the final ecdysis. A maximum incorporation rate is reached when the terminal oocytes are between 3.5mm and 5.5mm in length. After this stage there is a significant

decrease in the incorporation rate until the lowest level is reached when the eggs move into the oviducts (Hill, 1965). Thus in the normal female desert locust the incorporation of glycine into the fat body proteins follows a cyclical pattern which can be correlated with cycles of oocyte growth and neuroendocrine activity. When the neuroendocrine system activity produces and releases its factors, the rate of incorporation of glycine is high (Highnam, 1962a).

5.1.3.2. Role of endocrine hormones on protein synthesis in the course of sexual development of the female locusts

In the locust, oogenesis consists of four periods (Goltzene et al., 1978):

a) previtellogenesis

b) vitellogenesis I

c) vitellogenesis II

d) choriogenesis

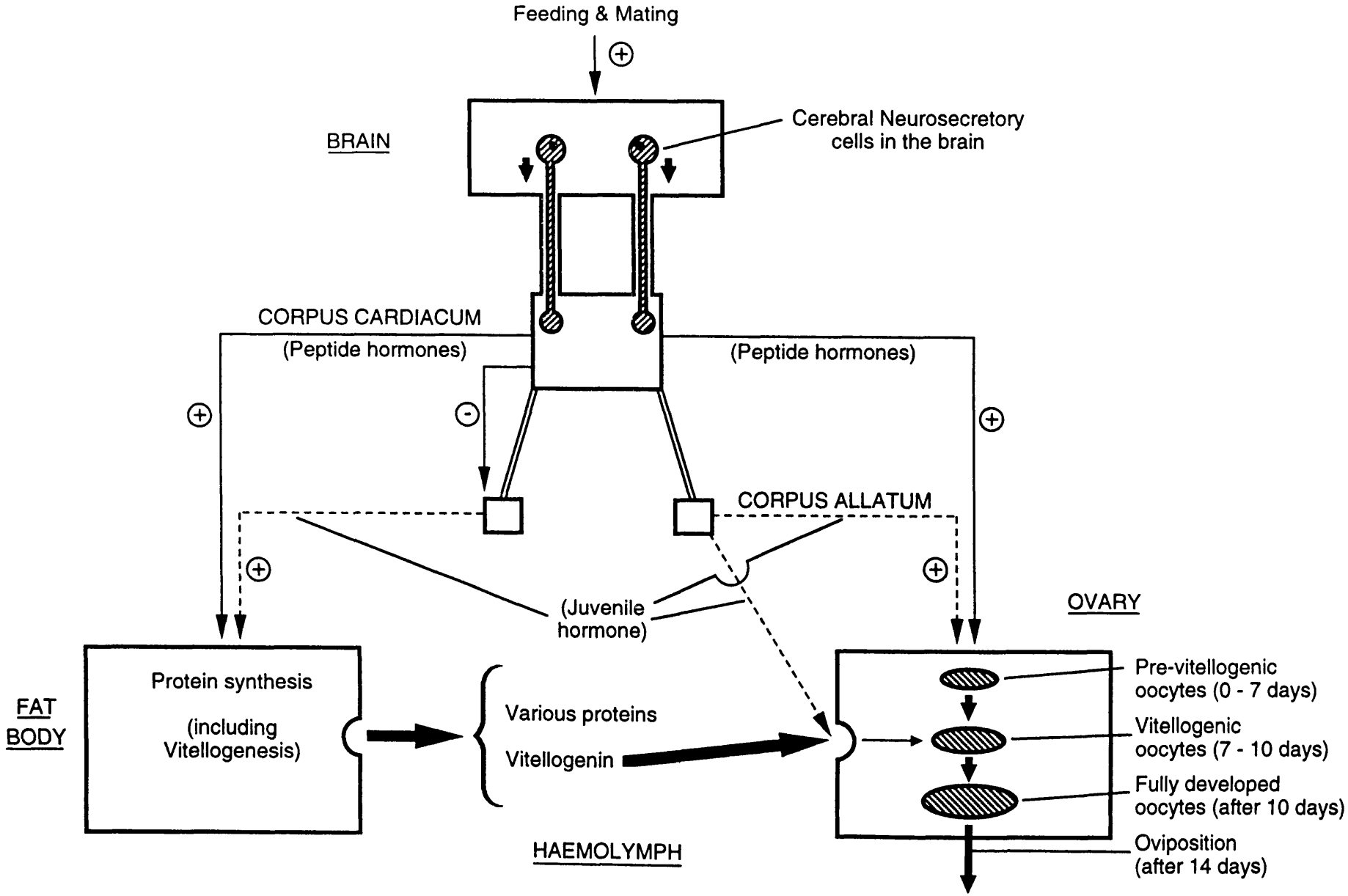
During previtellogenesis, the oocyte-synthesizing structures develop and make the translucent-white oocyte opaque. Vitellogenesis corresponds to the period of deposition of yolk proteins. The first phase of vitellogenesis (I), roughly correlated with the appearance of yellow oocytes, is characterized by a slow growth of the oocytes (oocytes length less than 2mm). During the second phase of vitellogenesis (II), the oocytes grow rapidly following an active uptake of yolk precursor proteins, from the haemolymph. During choriogenesis, the oocyte membranes are produced, and finally egg-laying occurs.

Oogenesis is controlled by both the pars intercerebralis and the corpora allata (Girardie et al., 1987a) (Fig. 5.1). The juvenile hormones produced by the corpora allata are essential for oocyte growth until full vitellogenesis II (Goltzene et al., 1978). This hormone controls the synthesis of vitellogenin in the fat body (Chen et al., 1979). In addition, juvenile hormone directly acts on the ovaries.

Fig.5.1 Schematic illustration of the endocrine control of reproduction in the locusts

⊕ : **Stimulation**

⊖: **Inhibition**


Feeding & Mating
BRAIN
Cerebral Neurosecretory cells in the brain
CORPUS CARDIACUM
(Peptide hormones)
(Peptide hormones)
CORPUS ALLATUM
(Juvenile hormone)
OVARY
FAT BODY
Protein synthesis
(including Vitellogenesis)
Various proteins
Vitellogenin
HAEMOLYMPH
Pre-vitellogenic oocytes (0 - 7 days)
Vitellogenic oocytes (7 - 10 days)
Fully developed oocytes (after 10 days)
Oviposition (after 14 days)

It determines the differentiation of the follicle cells during the first phase of vitellogenesis and the utilization of the perinuclear material in the ooplasm (Goltzene et al., 1978). The pars intercerebralis-corpora cardiaca neurosecretory system is required at the vitellogenesis I and at the end of the second phase of vitellogenesis when the vitelline membrane and chorion are produced by the follicle cells (Girardie et al., 1992).

It has been recorded in decapitated cockroaches that both juvenile hormone and neuroendocrine extracts stimulate ovarian protein formation (Keeley et al., 1985). Vitellogenesis requires the presence in the haemolymph of yolk protein precursors. Juvenile hormone appears to control transcription of two locust vitellogenin genes in the fat body (Wyatt, 1988). In addition to fat body, juvenile hormone has also ovarian targets in the locusts (Goltzene et al., 1978) where it appears to enhance vitellogenin uptake in isolated oocytes (Ferenz et al., 1981). The requirement for the neurohormone during the rapid growth of oocytes suggests that the neurohormone could also control the receptor-mediated uptake of vitellogenin similarly to juvenile hormone (Girardie et al., 1992).

It is well known that the synthesis of many ovarian proteins occurs outside the ovary (Telfer, 1965), and it is thought that the fat body is the site of this synthesis (Coles, 1964; Hill, 1965). It is likely that proteins are synthesised by the fat body, possibly stored there for some time, and then released into the haemolymph and taken up by the developing oocytes. There is considerable evidence to support this hypothesis in the desert locust (Hill, 1962; Hill, 1965; Highnam, 1963).

During the period before yolk begins to be deposited in the proximal oocytes, protein slowly accumulates in the fat body and haemolymph. No protein enters the ovary during this period. In each locust, during this period, about 20mg of protein accumulate in the haemolymph and about 5mg in the fat body. Studies on

the synthetic activity of the fat body during this initial (before 7 days) period suggest that little protein synthesis is taking place (Hill,1965).

5.1.3.3. Uptake of vitellogenin by the ovary

Of all the haemolymph proteins that are taken up in large amounts into the ovary, vitellogenin is the most extensively studied. Although in all the insect species studied, vitellogenin is synthesised by the fat body, the ovaries of *Drosophila melanogaster* (Bownes, 1986), *Dacus oleae* (Zongza et al., 1988) and *Leptinotarsa decemlineata* (Kanost et al., 1990) also have the capacity to synthesise this protein. In *Stomoxys calcitrans* the ovary is reported to be the sole source of vitellogenin (Chen et al., 1987). Vitellogenin is synthesised in large amounts in the female insects, but small amounts of protein have been reported in some male insects (Lamy, 1984; Hagedorn, 1985; Cardon et al., 1988).

Once vitellogenin enters the egg it is generally referred to as vitellin. Vitellogenin and vitellin are usually very similar in properties and composition (Mundall et al., 1979; Hagedorn et al., 1979; Kunkel et al., 1985) with only minor differences in electrophoretic mobilities under non-reducing conditions (Imboden et al., 1983). Vitellogenins isolated from a variety of insects consist of polypeptide sub-units which vary in number from one in *Apis mellifera* to four or more in *Periplaneta americana* , *Locusta migratoria* and *Rhodnius prolixus* (Harnish et al., 1982; Kunkel et al., 1985).

5.1.3.4. Vitellin and vitellogenin in the oocytes of the locusts

Locust vitellogenin and vitellin have been identified by electrophoretic and immunochemical techniques (Gellissen et al., 1976; McGregor et al., 1977; Chen et al., 1976). Although vitellogenin is only a small fraction out of many proteins present in the haemolymph, its uptake into the oocyte is highly selective (Kunkel et

al., 1985 and Ferenz, 1981) and probably involves micropinocytosis (Telfer et al., 1963; Bassemir, 1977). Oocyte growth depends on the uptake of vitellogenin. It is proposed that vitellogenin binds to specific receptors on the oocyte surface and the external membrane (oolemma) is invaginated to form oocytotes vesicles (Telfer, 1960).

5.1.4. Neurosecretory control of haemolymph protein during ovarian development in the locust

Ovarian development in the locust is controlled by the cerebral neurosecretory system (Highnam, 1962a, b) (Fig. 5.1). While the terminal oocyte of the ovary are developing, neurosecretory hormones are synthesized and released into the haemolymph but there is no release material before and after this period. In females reared in the absence of mature males, neurosecretory material is not initially released, and it accumulates in the corpora cardiaca. In these insects, maturation is delayed. In females reared with mature males no accumulation of neurosecretory material occurs and maturation proceeds rapidly (Highnam et al., 1962). Removal of median neurosecretory cells by cautery, or cardiacectomy, prevents ovarian development, and reimplantation of neurosecretory cells allows ovarian development to proceed (Highnam, 1962 a,b).

It has been well established that the role of juvenile hormone (JH) is to prevent metamorphosis in immature insects, thus determining the nature of the molt induced by ecdysone. In the locusts, juvenile hormone-dependent vetellogenin synthesis is normally limited to the fat body of adult females as described in above. After allatectomy, either by surgery or by chemical means, vitellogenin synthesis can be induced by treatment with JH or an analog such as methoprene. It has been recorded that the stability is higher in methoprene than in natural JH (Henrick et al., 1973). In the adult female locust, the fat body becomes competent to synthesize

vitellogenin in response to injected JH analogue on days 3-5, about 3 days before synthesis is normally turned on by JH released from corpora allata (Johnson et al., 1975; Rembold, 1981). JH stimulation of the adult female fat body is highly specific for vitellogenin synthesis. The fat body of adults of both sexes also shows a relatively low level stimulation of non-vitellogenin protein synthesis (Gellissen et al., 1981).

Adult female locusts may be allatectomized chemically by treatment with ethoxyprecocene. The precocenes are naturally occurring chromenes, found in plants in the genus *Ageratum* (Bowers. 1976). In sensitive insects the precocenes have been shown to exert a cytotoxic action on the insect corpus allatum, the endocrine gland responsible for JH synthesis and secretion, thereby inducing a state of hormone deficiency in the treated animal, identical to that produced by surgical allatectomy (Bowers et al., 1977; Pener et al., 1978; Bowers et al., 1980).

5.1.5. Incorporation of ^{35}S-cysteine into the corpus cardiacum and the brain of the locust

The neurosecretory proteins are rich in the sulphur-containing amino acid, cysteine (Sloper, 1957). Therefore ^{35}S-cysteine has been used as radiolabelled tracer to estimate the incorporation level of neurosecretory protein (Mordue et al. 1973; Highnam et al., 1974; Giradie et al., 1985; Subrahmanyam et al., 1989b). The radioactivity in the storage lobes of the corpora cardiaca arises in three ways (Mordue et al., 1973, Girardie et al., 1987a). First, a very rapid labelling due to chemical interchange between the labelled and unlabelled sulphur, molecular interchange between the amino acid and its counterpart in the neurosecretory protein, and adsorption of the amino acid on the structural tissues of the gland; second, the transfer to the corpora cardiaca of neurosecretory material which has become labelled during its production in the cerebral neurosecretory

cells; and third, the labelled protein which is synthesised by the glandular lobes of the corpora cardiaca . The label attributed to the former is in a state of dynamic equilibrium with the label in the haemolymph, and it is presumed that this equilibrium is attained very rapidly because of the intimate relationship between the haemolymph and the corpora cardiaca.

5.1.6. Non-protein metabolism of ^{35}S-cysteine in the locust tissues

Metabolism of ^{35}S-cysteine in the tissues of the locust has been examined (Whitton, 1987). In order to estimate potential ^{35}S-cysteine-containing compounds that might interfere with quantitative estimating of the specific activity of taurine and its precursors, ^{35}S-cysteine was assessed using autoradiography. It was found that most of the injected ^{35}S was present as oxidised glutathione which interferes with the separation by TLC of other small peptides. This problem was solved following oxidation and derivatization with N-ethylmaleamide (Whitton, 1987).

5.1.7. Effect of starvation on the neuroendocrine system

In *S. gregaria* starvation greatly retards oocyte growth and has a marked effect upon the content of material in the neurosecretory system (Highnam et al., 1966). When females with developing eggs are starved, the autoradiographic studies have shown that stainable material quickly accumulates in the corpora cardiaca and the brain . Release of neurosecretory material is reduced by starvation although some synthesis can still occur to bring about the accumulation of material within the cells and corpora cardiaca (Mordue et al., 1973).

5.1.8. Neurosecretory peptides and protein in the corpora cardiaca

Neurosecretory cells of the corpora cardiaca release/produce different types of peptide hormones. A hyperglycaemic hormone, secreted by the

neurosecretory cells of the corpus cardiacum, controls the quantity of blood trehalose during flight (Girardie et al., 1987a). Adipokinetic hormone is released from the corpora cardiaca and stimulates the breakdown of fat body triglyceride (Girardie et al., 1987b). Some of the other neurosecretory hormones which are released from the corpus cardiacum relate to oocyte development and starvation. It has been established that three major cysteine-rich proteins (monomer, dimer, and trimer) are found in the 70% aqueous methanol-soluble fraction of corpora cardiaca of the locust. The labelling by ^{35}S-cysteine of the three proteins, formed from two polypeptide chains linked by disulfide bonds, confirms that the three molecules contain cysteine (Girardie et al., 1985). The incorporation of cysteine into the dimer, in both intact locusts and locusts in which the corpora cardiaca are disconnected from the brain before injection of the radioisotope, demonstrates that the dimer is not a secretory product of the brain. This protein is synthesised by the glandular lobes of the corpora cardiaca and named as 'cardiacin' (Girardie et al., 1987a).

The trimer is detected only in the median area of the brain. It increases substantially in amount when transport of neurosecretory brain material is disrupted by disconnection of the corpus cardiacum from the brain. Thus the trimer is the only identified labelled neurosecretory protein synthesized in the brain and then transported to the corpus cardiacum via the nerves. This neurosecretory protein was named 'neuroparsin A' (Girardie et al., 1987a).

The monomer is never detected in the three areas of the brain, even for locusts in which the corpus cardiacum was disconnected from the brain before injection of the radiolabelled cysteine. The monomer appears in the corpus cardiacum when neuroparsin A is transported into the corpus cardiacum. The monomer certainly results from a transformation of neuroparsin A at the median neurosecretory endings. It was named 'neuroparsin B' (Girardie, 1987a).

Transformation occurs over a short time since neuroparsin B is present by 4 hours after the injection of radioisotope. Neuroparsin B is not detected in the locusts in which the corpus cardiacum was disconnected from the brain in which the neuroparsin A is present at high concentration.

It is clear from previous work that neurohormones are synthesised by both the corpus cardiacum and the brain.

Keeping this in view, the present study was aimed at studing the effect of azadirachtin on the incorporation of radiolabelled amino acids into the protein of the identified locust tissues.

The aim of the work reported in this chapter was to examine the incorporation of radio-labelled amino acids into the proteins and peptides of a number of identified tissues to see if, in fact, azadirachtin can be shown to have general effect on protein biosynthesis in the locust. For this purpose only maturing adult female locusts were used, at the time of their most vigorous protein synthesis: i.e.while they are forming oocytes. The tissues examined for this purpose were fat-body, haemolymph and ovary. As has been already discussed, these tissues are linked in the formation, transport and uptake of vitellogenin and possibly other proteins. The effects of azadirachtin on the biosynthesis of proteins by the mid-gut was also examined, as any reduction of activity of proteolytic and other digestive enzymes would be expected to have profound effects on the growth on the insects The tracer used was tritiated glycine, as this had been previously found to be a suitable amino acid for this purpose (Hill, 1965).

It was also intended to examine more exactly the effect of azadirachtin on the synthesis of possible neuropeptide material by the brain and corpus cardiacum, as this might be expected indirectly to affect a wide range of metabolism including protein biosynthesis in the locust tissues. For this purpose 35S cysteine was used,

following the work of Mordue et al. (1973) who showed that this amino acid was abundant in neurosecretory peptides.

The work *in vivo* was then followed by experiments done *in vitro* with all the tissues named examined separately, in an attempt to distinguish between direct and indirect effects of the azadirachtin.

5.2. Methods and materials

5.2.1. Insects

Adult female *S. gregaria* (7 days after final ecdysis) were used for all experiments and were fed and maintained under the laboratory conditions described in Chapter 3.

5.2.2. *In vivo* experiments

5.2.2.1. Azadirachtin treatment

The experimental locusts were injected with a dose of 3μg azadirachtin/g body weight and the control insects starved for 12 hours before the experiment, received the same amount of 10% ethanol/g body weight as the azadirachtin-treated locusts.

5.2.2.2. Incubation media

A preliminary experiment was carried out comparing a simple insect saline with insect cell culture medium to select the best medium for incorporation of radiolabelled amino acids into acid precipitable protein.

a. Insect saline

Saline containing: KCl, 10mM; NaCl, 140mM; $CaCl_2$, 2mM; glucose, 10mM; and MOPS buffer, 10mM; pH 6.9, was used in this experiment as a simple locust physiological medium.

b. Insect cell culture medium

The cell culture medium contained all the amino acids, inorganic salts and other components which are present in the haemolymph of insects (See appendix).

5.2.2.3. Application of radiolabelled amino acids

Both ^{3}H-glycine and ^{35}S-cysteine were obtained from Amersham International, Aylesbury, Bucks. The specific activity of the ^{3}H-glycine and the ^{35}S-cysteine were 21Ci/mmol and 1300Ci/mmol respectively. Each radiolabelled amino acid was dissolved in insect saline to give a final concentration of 1μCi/ 10μl. Each amino acid (2μCi/gm) was injected into each locust 12 hours after injection of azadirachtin. Injections were made through the abdominal intersegmental membrane using a Hamilton 25μl syringe.

5.2.2.4. Loss of radioactivity from the haemolymph

Loss of radioactivity from the haemolymph was measured over an 8 hour period by taking 1μl blood samples from the base of the hind coxa of the locust using 5μl micropipettes at different times after injection of the label and the radioactivity within each sample was measured by the liquid scintillation techniques described in Chapter 3.

5.2.2.5. Excretion of injected radioactivity

The excretion of azadirachtin-treated females was compared with normally-feeding and starved females. Individual locusts were kept in separate beakers for periods up to 7 days. During this time only azadirachtin-treated locusts and one control group were allowed access on fresh cabbage leaves and the other control group were kept starved. Faeces were collected at the end of the each 24 hour period and air dried overnight at room temperature before being weighed. The radioactivity of the samples was analysed as described in Chapter 3.

5.2.2.6. Incorporation of radioactivity into total proteins of whole locusts

Initially, the rate of incorporation over six hours was measured in both control and azadirachtin-treated locusts. The radioactivity in the whole locust was analysed in the following manner: individual locusts were anaesthetized in an atmosphere of CO_2 at different times after injection of the labelled amino acids and were then frozen in liquid N_2, before grinding to a uniform powder in a mortar pre-cooled with liquid N_2. The pooled samples were homogenised for 30s in 0.5ml of 5% trichloroacetic acid (TCA) using an ultrasonic microprobe. Samples were kept deep frozen in 5% TCA until processing.

The homogenate was centrifuged for 5min at 10,000g and the pellet washed three times in 5% TCA . This process removed all radioactivity not incorporated into the proteins. The protein pellet was redissolved in 200 µl of 0.1M NaOH containing 0.1% w/v SDS overnight at 30°C, and 100µl of the sample was used to estimate the radioactivity by scintillation counting. The rest of the sample was used for protein estimation by the method of Lowry *et al.* (1951), against a standard of bovine serum albumin, and then the specific activity of the tissue was estimated with reference to the protein content.

5.2.2.7. Incorporation of ^{3}H- glycine into specific tissues

The locusts were anaesthetized under CO_2 at different times after injection of the radioactive amino acid and the tissues were quickly dissected out under ice-cold insect saline. They were washed in fresh insect saline before being placed in 0.5ml of 5% TCA. The samples were homogenized as described 5.2.2.6. Tissues examined were in the fat body, the haemolymph, the ovary and the mid gut.

5.2.2.8. Incorporation of ^{35}S-cysteine into the brain and corpus cardiacum

Each locust was injected with 2μCi ^{35}S-cysteine / g and the rate of incorporation was measured 4 hours after injection. The heads were removed and the endocrine system was quickly dissected out under ice-cold saline. The brain and corpus cardiacum were isolated free of fat body and were washed with fresh insect saline before further analysis. Pooled samples from batches of 4 locusts were homogenized for 30sec in 500μl of 70% v/v aqueous methanol using an ultrasonic microprobe. The suspension was centrifuged at 10,000g for 5min and the pellet washed twice with 5% TCA. The pellet was dissolved in 100μl 0.1M NaOH containing 0.1% w/v SDS overnight at 30°C. The supernatant was freeze-dried and redissolved in 100μl of 70% v/v aqueous methanol. The samples (50μl) of the supernatant and the redissolved protein were taken for estimation of radioactivity by scintillation counting. The rest of the protein-containing pellet was used to estimate the quantity of protein and the quantity of labelled compound in the tissue was estimated with reference to the protein content. Observations were repeated four times.

5.2.2.9. Effect of extracts from neuroendocrine tissues on the protein turnover

5.2.2.9.1. Preparation of corpus cardiacum extraction

Adult female locusts (7days after final ecdysis) were anesthetized under CO_2 and the heads removed. The corpus cardiacum was dissected out, free of the fat body, under saline, and placed in 100µl of insect saline. The gland was homogenised for 30sec using an ultrasonic microprobe.

5.2.2.9.2. Effect of extracts of corpus cardiacum on the rate of incorporation of ^{3}H-glycine into the specific tissues of the locusts.

The locusts were head-ligatured 12 hours before injection of the ^{3}H-glycine and only the experimental locusts were injected with azadirachtin along head-ligation. The incorporation levels of the tissues were measured 2 hours after injection of the ^{3}H-glycine in both control and azadirachtin-treated locusts. Tissues were analysed as described in 5.2.2.6.

The above experiment was extended as follows to observe the effect precisely: The crude extract of equivalent single corpus cardiacum as described in 5.2.2.9.1. was injected into each ligatured locust 3 hours before injection of ^{3}H-glycine, and then the rate of incorporation was measured.

To investigate whether there is any simple physical effect on uptake of amino acids due to ligaturing, the clearance of ^{3}H- glycine from the haemolymph was measured in both control and azadirachtin-treated locusts as described in 5.2.2.4.

5.2.3. *In vitro* experiments

5.2.3.1. Incorporation of ^{3}H-glycine into specific tissues of the locust

The locusts were anaesthetized under CO_2 and dissected in insect saline. The fat body was removed from the insects and freed from the ovary and tracheae. The sheets of the fat body were preincubated separately in 1ml of insect saline at 30°C for 30min, and then 1μCi of ^{3}H-glycine was added to the medium. Incorporation of the radiolabel into protein was measured after 1, 2 and 4 hours as described in 5.2.2.6.

Subsequently, the experiments were done using the fat body, ovary, and mid gut and the incorporation rate was measured 2 hours after application of ^{3}H-glycine into the medium. The control experiment was carried out with the locust tissues which were preincubated for 30min. The tissues of experimental locusts were preincubated with 12μg of azadirachtin in the same way as the control experiment. Then the theoretical concentration of azadirachtin in the medium was same as the *in vivo* experiment. The insect saline was maintained at a constant temperature of 30°C and aerated throughout the experiment. Each experiment was repeated four times.

5.2.3.2. Effect of neurohormonal extract on incorporation of ^{3}H-glycine into the fat body

Insect saline (1ml) containing the extract of a single corpus cardiacum as described in 5.2.2.9.1. was used as the medium for incubation of tissues and then the rate of incorporation was measured as described in 5.2.2.9.2. The experiments were carried out for both control and azadirachtin-containing media.

5.2.3.3. Effect of boiling on the corpus cardiacum extraction

In an attempt to determine the nature of the neurohormones, the corpus cardiacum extract was boiled for different times (5min and 10min) and then added to the 1ml of insect saline and the experiment conducted as above.

5.2.3.4. Incorporation of ^{35}S-cysteine into the brain and corpus cardiacum

Brains and the corpora cardiaca were dissected out separately and placed in 1ml of the insect saline. Tissues from a batch of 12 locusts was pooled for each experiment. Each sample was preincubated for 30min before adding 1μCi of ^{35}S-cysteine. The rate of incorporation was measured after 4 hours. The insect saline was maintained at a constant temperature of 30^{o}C and aerated throughout the experiment.

The samples were washed in fresh insect saline before being analysed and then they were homogenised in 500μl of 70% methanol and centrifuged for 5min at 10,000g. The supernatant was freeze-dried and redissolved in 50μl of 70% aqueous (v/v) methanol. The analysis of pellet was as described in 5.2.2.8.

5.2.3.5. Direct effect of azadirachtin on uptake of ^{35}S-cysteine into the brain and corpus cardiacum

A batch of four preincubated brains and the corpora cardiaca was placed in 1ml of insect saline which contained 0.25μCi of ^{35}S-cysteine and samples of the medium (1μl) were taken out to measure the radioactivity at different times. To investigate the effect of azadirachtin on the uptake the tissues were preincubated with 12μg of azadirachtin and the radioactivity in the medium was measured as same way as the control experiment.

5.2.3.6. Metabolism of ^{35}S-cysteine in the brain and corpus cardiacum

5.2.3.6.1. Thin Layer Chromatography (TLC)

The tissue extracts in 70% methanol were chromatographed on a 20x20cm cellulose TLC plate. The plate was double-developed in butanol : acetic acid : water (11: 6: 3 v : v : v). The position of the radio-labelled amino acids contained in the extract was identified using following standards ; a) glutathione (oxidised) b) cysteic acid c)cysteine d) cystine e) cysteine sulphinic acid f) glutathione (reduced). After resolution, these amino acids were visualised by dipping in 1% ninhydrin in acetone and heating for 5min at 100^{o}C. The bands (1.0cm) were scraped from the plate and then they were transferred to scintillation vials for counting, so that the entire plate was analysed for radioactive spots.

5.2.3.6.2. Derivatization of thiol groups

The extracts of brain and corpus cardiacum were reduced using 2mM sodium borohydride and then reacted with 10mM N- ethylmaleamide (NEM) to form NEM derivatives. The standards were also converted to a NEM derivatised form and analysed by TLC as above.

5.2.3.6.3. Exclusion chromatography

Exclusion chromatography was carried out to separate the low (M_r<1300) and high (M_r>1300) molecular weight peptides of the 70% methanol-soluble fraction of both the brain and corpus cardiacum.

5.2.3.6.3.1. Preparation of the column

Sephadex G-10 was used as the stationary phase and 70% methanol - water was used as the solvent. To obtain good separation and satisfactory flow rates the gel was allowed to swell in excess solvent and left to stand for 2 hours. A syringe (1ml) was used as a column and the material was packed without any air bubbles.

5.2.3.6.3.2. Preparation of the sample

The pooled samples of the 70% methanol-soluble fraction of both brain and corpus cardiacum were used separately First they were reduced with sodium borohydride and then converted to the NEM derivatives. Two coloured markers with different molecular weights were used to identify the fractions. They were vitamin B_{12} (Cyanocobalamin), red in colour, with molecular weight (M_r) 1333 and 2,4,DNP-L-lysine hydrochloride, yellow in colour, with molecular weight (M^r) 348. A sample (5µl) of each marker (10mM in 70% methanol) was mixed with 50µl of the extract and this sample was added into the top of the column and eluted with 1ml of 70% methanol.

5.2.3.7. Effect of azadirachtin on juvenile hormone-induced protein synthesis in the fat body *in vitro*

To destroy the corpora allata, 0.5mg of ethoxyprecocene (Sigma) in 5µl of acetone was applied topically on the neck membrane of adult female locusts within 12 hours after the final ecdysis (Chinzei et al, 1982). A week later, the fat body was dissected out and incubated in 2ml of the insect cell culture medium containing 60µg of methoprene for 24hours (Wyatt et al, 1976). Then the fat body was placed in 1ml of insect saline with 1µCi of ^{3}H-glycine and incubated for 2hours. The incorporation of radiolabelled amino acids into protein was measured

as described in 5.2.2.6. To check the effect of azadirachtin the fat body was incubated with azadirachtin and methoprene for 24 hours and the experiment was continued as same way as the control.

5.3. RESULTS

5.3.1. Loss of radioactivity from the haemolymph

The clearance of ^{3}H-glycine and ^{35}S-cysteine from the haemolymph were each measured over 8 hours. Both amino acids showed a similar clearance pattern. The percentage of radioactivity remaining in the haemolymph is shown in Fig. 5.2. More than 60% of the radioactivity was lost from the haemolymph within the first 30min of injection, and, 8 hours after injection, only 30% of the total radioactivity was detectable. In the azadirachtin-treated locusts, the clearance of labelled amino acid from the haemolymph showed the same rate as control locusts. According to the Fig.5.2 the results suggest uptake of label is exponential, but that there is more than one mechanism.

5.3.2. Excretion of injected radioactivity

The excretion of ^{35}S-cysteine from azadirachtin-injected females was compared with 12 hour starved locusts and normal fed locusts. Fig.5.3 shows the excretion profile of injected radiolabelled cysteine over 2 days. The excretion pattern of normal locusts was different from azadirachtin-injected locusts and starved locusts. Only 40% of the applied radioactivity was excreted during the first 24 hours in normal locusts but both in the starved and azadirachtin treated locusts more than 60% of the total radioactivity was excreted.

Fig.5.2. Clearance of radioactive amino acids from the locust haemolymph

Doses of 2μCi of ^{35}S-cysteine (●) and ^{3}H-glycine (○) were injected separately into the haemolymph of the locust at zero time and samples taken as described. The points are the mean of 4 locusts, and the vertical bars indicate the standard deviation. The upper curve indicates the semilogarithmic representations of the uptake of ^{35}S-cysteine (●)and ^{3}H-glycine (○) from the locust haemolymph.

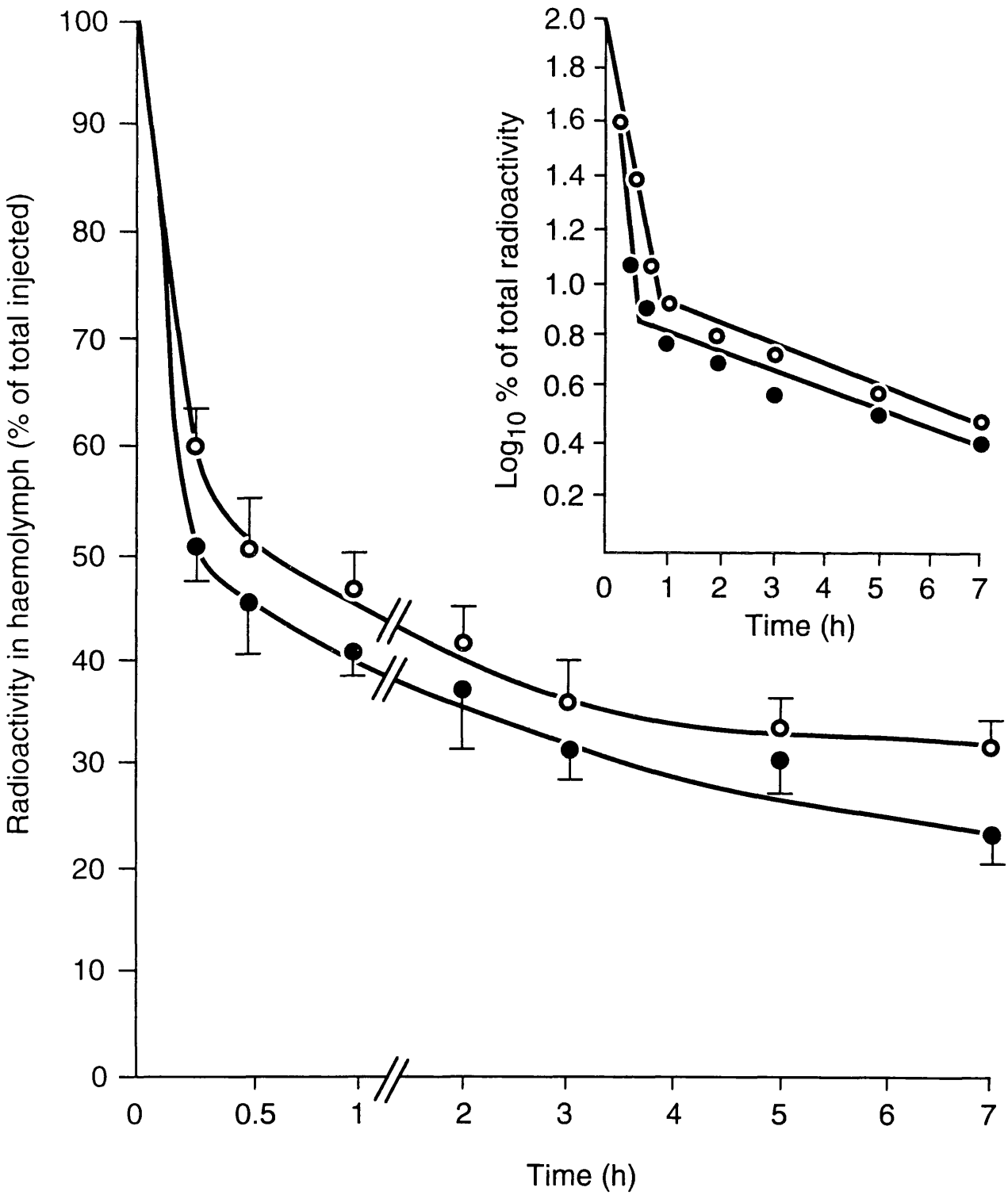
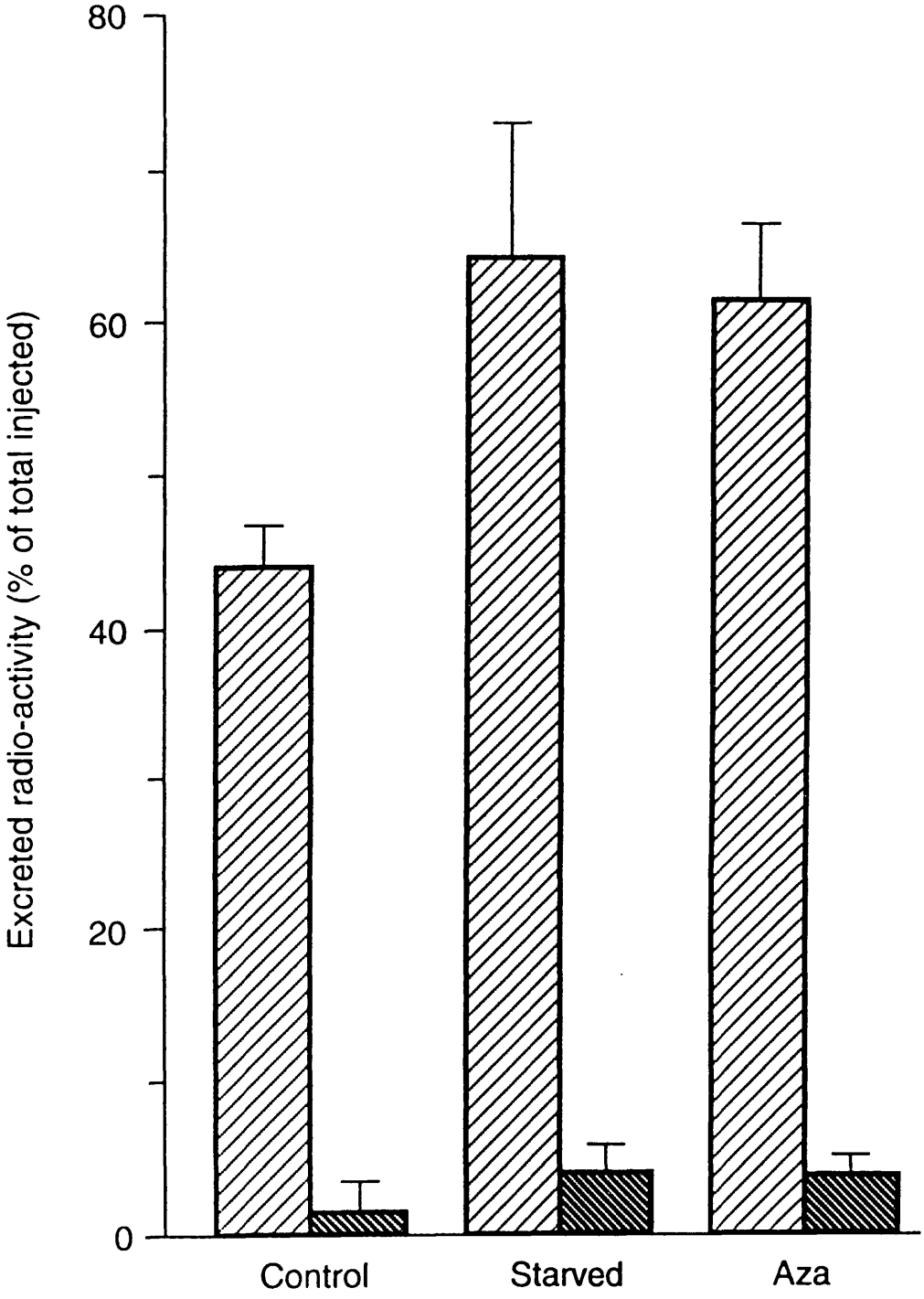

Radioactivity in haemolymph (% of total injected)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
2
3
4
5
6
7
Time (h)
Log10 % of total radioactivity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
Time (h)

Fig.5.3. Excretion of radio-activity from the locust *S. gregari* after injection of a dose of ^{35}S-cysteine.

The results are the means from 4 locusts, each of which received 2µCi c ^{35}S-cysteine with a specific activity of 1300Ci. mmol^{-1}, and were thereafte kept separately. Only control and azadirachtin-treated locusts were allowe access with fresh food and water. Bars indicate the standard deviation fro the mean.


-24 hours after injection


-48 hours after injection


80
60
40
20
0
Excreted radio-activity (% of total injected)
Control
Starved
Aza

Very little radioactivity was excreted after 24 hours, although there was a small constant, daily excretion, about 2% of the total. The remainder was retained in the body. The results were very similar to each other in all three groups after 24 hours.

5.3.3. Incorporation of radiolabelled amino acids into total protein in whole locusts

The rate of incorporation over 6 hours of ^{3}H-glycine and ^{35}S-cysteine into the proteins of the 7 day old females adult locust is shown in Fig. 5.4. This experiment was carried out using whole locusts without discriminating between individual tissues, and it was carried out as preliminary experiment which demonstrated a linear incorporation rate of radiolabelled amino acids into the locust tissue proteins during the first hour after injection, followed by the attainment of an equilibrium level. The patterns of incorporation of both amino acids in whole locusts were very similar to each other, although, specific activity of cysteine was higher than glycine.

The incorporation rate was significantly greater in control locusts than in azadirachtin-treated locusts. Both amino acids showed a 50% inhibition of the levels of incorporation due to azadirachtin.

5.3.4. Incorporation of ^{3}H-glycine into specific tissues of the locusts

Following the initial experiment, the rate of incorporation of ^{3}H-glycine into the fat body, the haemolymph, the ovary, and the mid gut were measured over 6 hours to investigate precisely the effect of azadirachtin on individual tissues.

Fig.5.4. The time course of incorporation of ^{35}S-cysteine and ^{3}H-glycine into the proteins of whole locusts.

The time course of incorporation of ^{35}S-cysteine (●) and ^{3}H-glycine (■) into the whole locusts after injection of 2μCi/g body weight into the haemolymph of 7 days old (after the final ecdysis) female *S. gregaria* were measured. The experimental locusts (----) were injected with 3μg azadirachtin/g 12 hours before injection of radiolabelled amino acid and the control locusts (▬) received same amount of 10% ethanol/water. The values represent the mean (±SD) of 4 locusts. The effects of azadirachtin were significantly different ($p<0.01$) from the control at each time recorded.

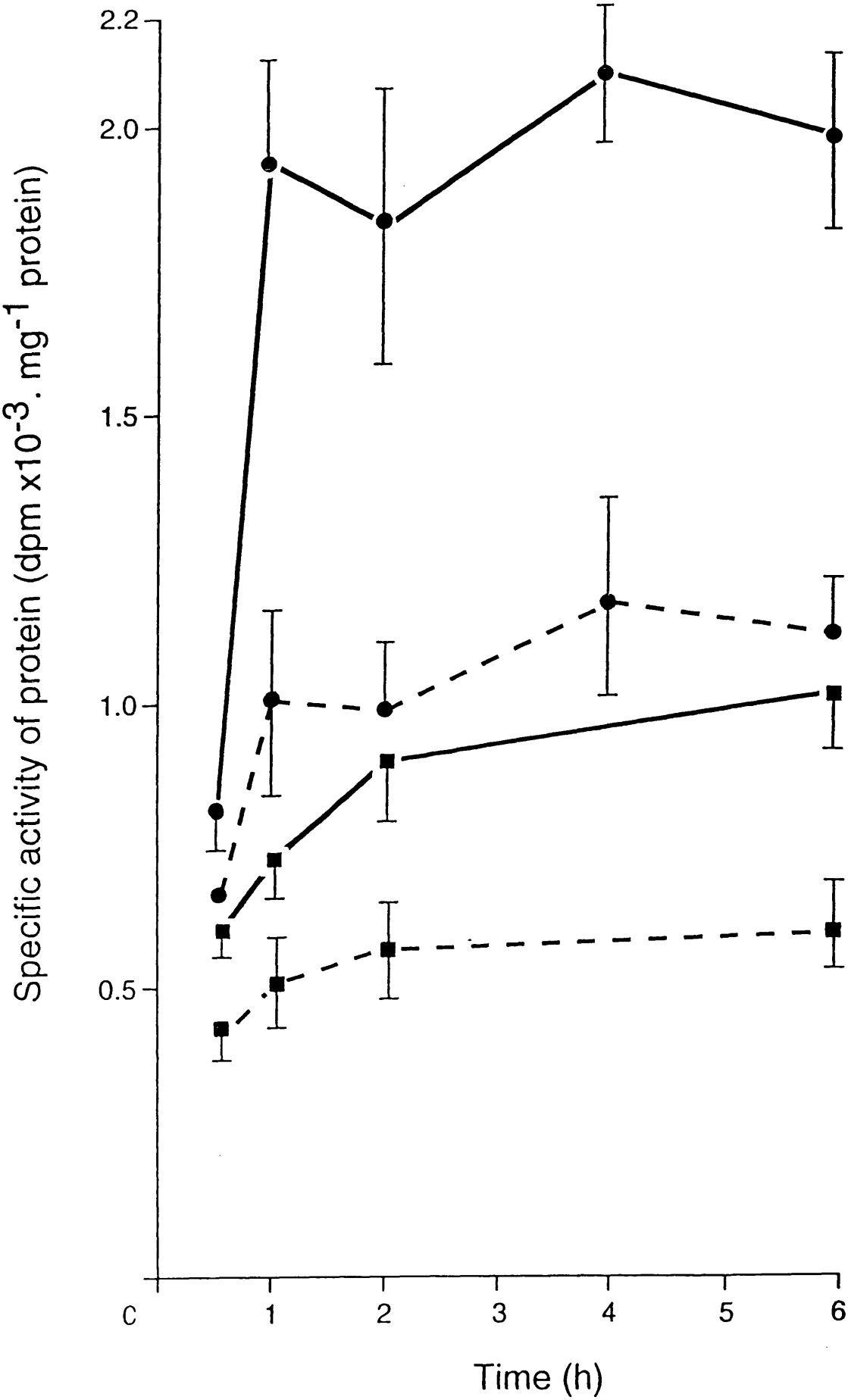
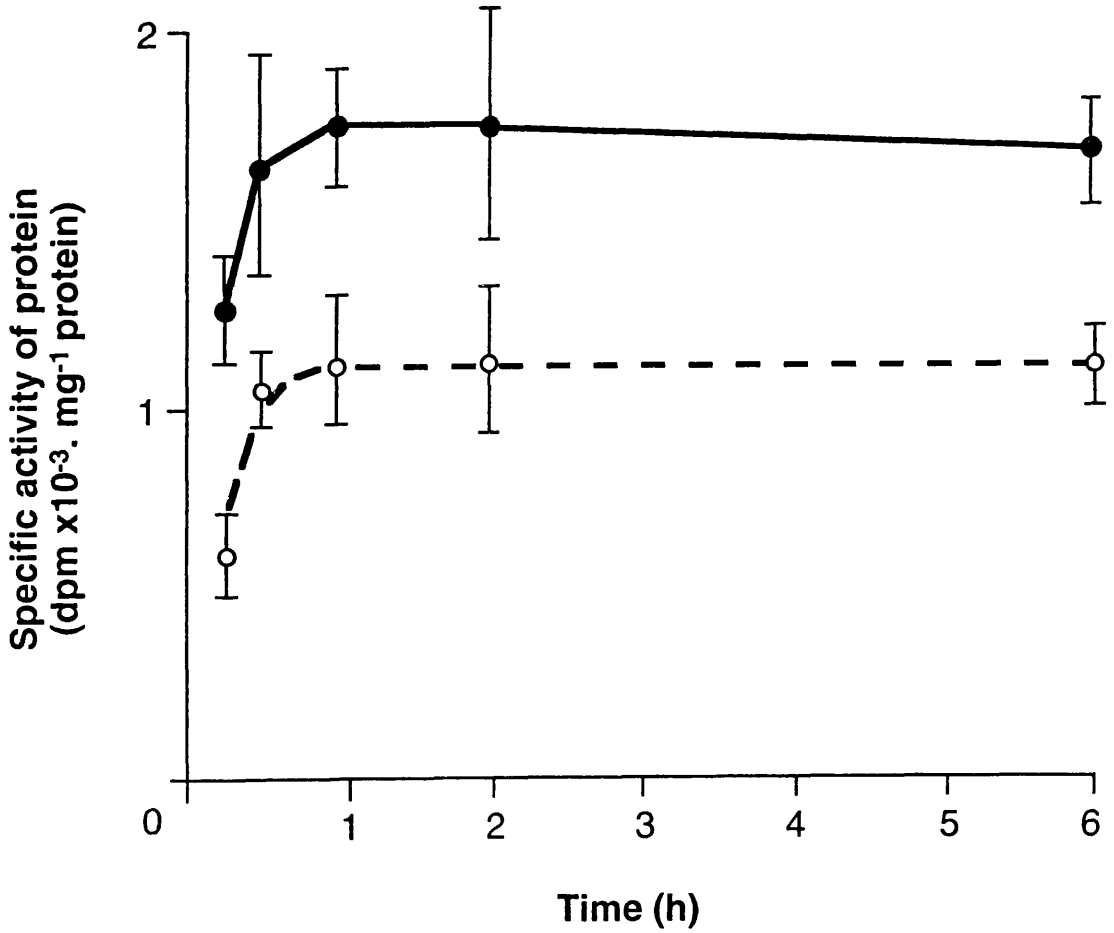

2.2
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
Specific activity of protein (dpm x10-3. mg-1 protein)
Time (h)

Fig.5.5. The incorporation of ^{3}H-glycine into the proteins of fat body

The time course of incorporation of ^{3}H-glycine into the fat body protein of the locust after injection of 2μCi/g body weight into the haemolymph of 7 days old (after the final ecdysis) female *S. gregaria.* was measured. The experimental locusts (o) were injected with 3μg azadirachtin/g 12 hours before injection of radiolabelled amino acid and the control locusts (●) received same amount of 10% ethanol water. The values represent the mean (±SD) of 4 locusts. The effects of azadirachtin were significantly different ($p<0.01$) from the control at each time recorded.


Specific activity of protein
(dpm x10-3. mg-1 protein)
2
1
0
1
2
3
4
5
6
Time (h)

5.3.4.1. Incorporation of ^{3}H-glycine into the proteins of fat body

Fig. 5.5 shows the time course of incorporation of ^{3}H-glycine into the fat body of the 7days old female adult locust. Both groups showed maximum incorporation level at 1 hour after injection and in the azadirachtin-injected locusts, the incorporation of radiolabelled amino acid into the protein was not as efficient as in the control locusts. At the end of the one hour period the incorporation levels of azadirachtin-treated locusts was 43% that of control locusts. These values were significantly different from each other.

5.3.4.2. Incorporation of ^{3}H-glycine into the haemolymph proteins

The incorporation of ^{3}H-glycine into the haemolymph proteins was measured over 6 hours, and the results of the time course of incorporation of ^{3}H-glycine into the haemolymph proteins of the locust are shown in Fig. 5.6. The level of incorporation was very slow compared to the fat body. Both control and azadirachtin-treated locusts showed a level of incorporation which increased gradually with the time. The incorporation level did not reach to equilibrium 1 hour after injection as in the fat body. The control locust showed a higher level of incorporation than the azadirachtin-treated locusts but these differences did not show any statistical significance.

5.3.4.4. Incorporation of ^{3}H-glycine into the proteins of ovary

Fig.5.7 shows the incorporation of ^{3}H-glycine into the proteins of ovary of adult female locusts 7 days after final ecdysis. The level of incorporation of the ovary was higher than the fat body and the haemolymph. The incorporation rates of control locusts were faster than those of the azadirachtin-treated locusts.

Fig.5.6. The incorporation of ^{3}H-glycine into the haemolymph proteins

The time course of incorporation of ^{3}H-glycine into the haemolymph protein of the locust after injection of 2μCi/g body weight into the haemolymph of 7 days old (after the final ecdysis) female *S. gregaria.* was measured. The experimental locusts (○) were injected with 3μg azadirachtin/g 12 hours before injection of radiolabelled amino acid and the control locusts (●) received same amount of 10% ethanol water. The values represent the mean (±SD) of 4 locusts.The effects of azadirachtin were not significantly different from the control experiment.

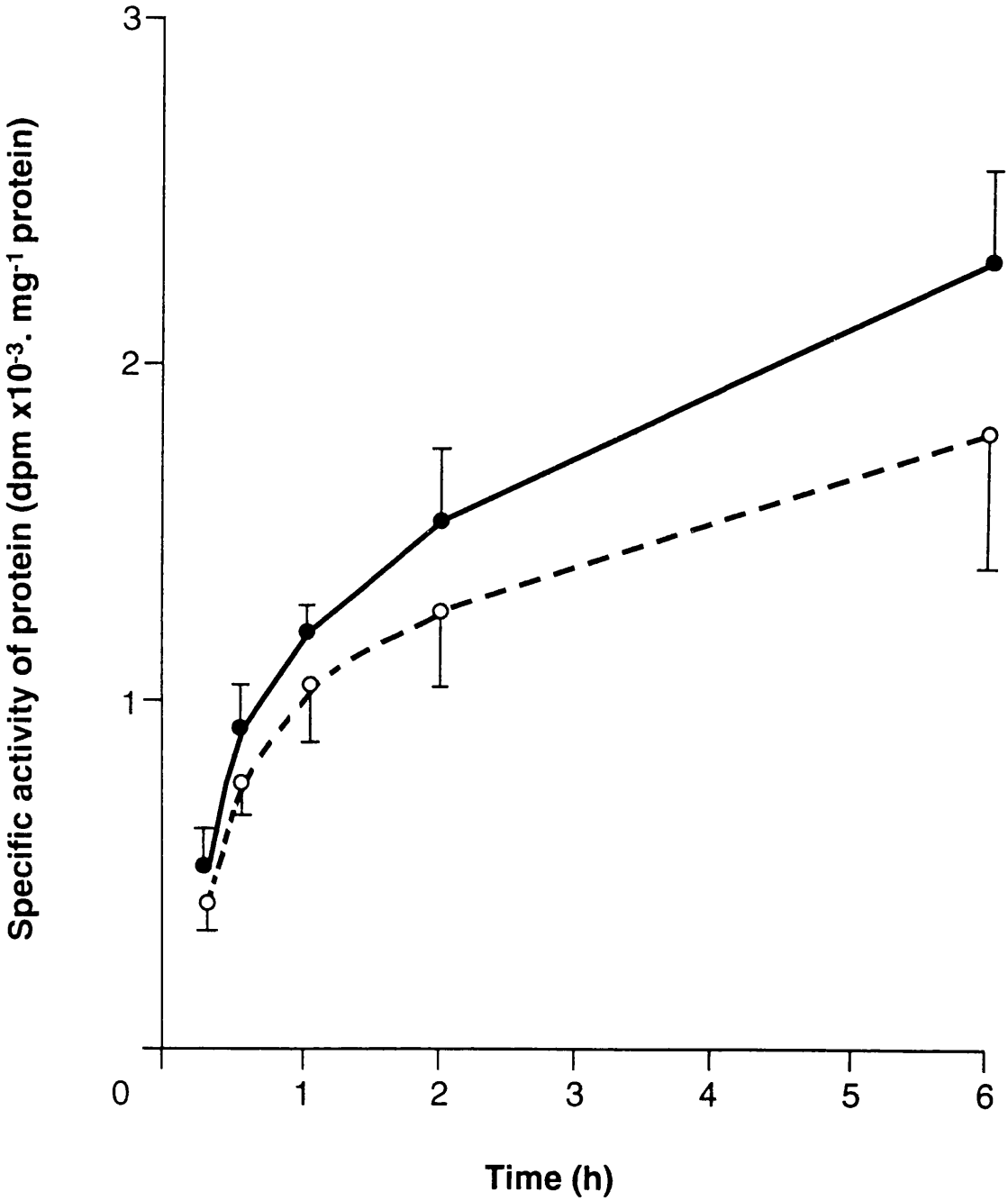
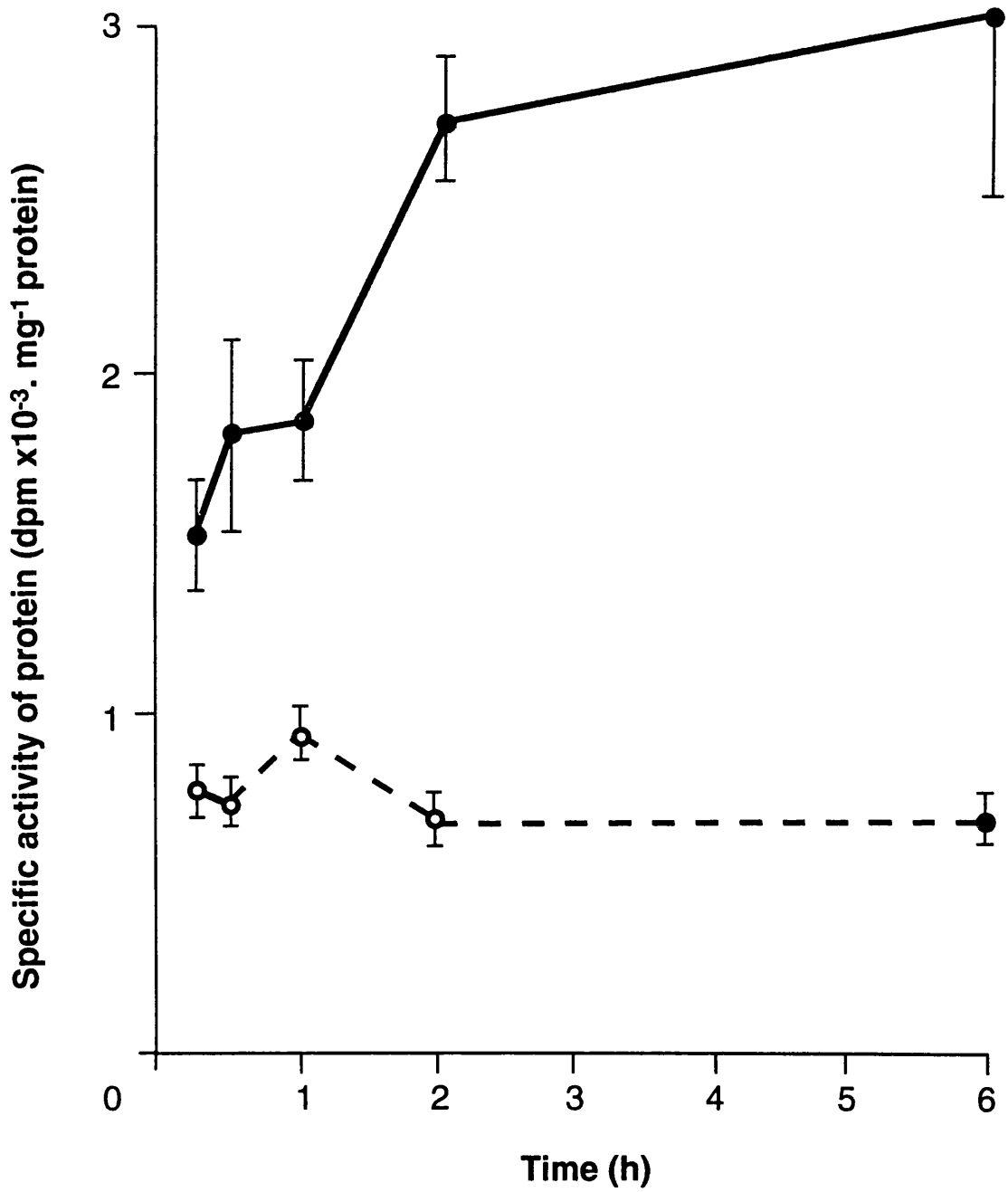

Specific activity of protein (dpm x10-3. mg-1 protein)
3
2
1
0
1
2
3
4
5
6
Time (h)

Fig.5.7. The incorporation of ^{3}H-glycine into the proteins of ovary

The time course incorporation of ^{3}H-glycine into the ovary protein of the locust after injection of 2μCi/g body weight into the haemolymph of 7 days old (after the final ecdysis) female *S. gregaria.* was measured. The experimental locusts (○) were injected with 3μg azadirachtin/g 12 hours before injection of radiolabelled amino acid and the control locusts (●) received same amount of 10% ethanol water. The values represent the mean (±SD) of 4 locusts. The effects of azadirachtin were significantly different from the control experiment.


Specific activity of protein (dpm x10-3. mg-1 protein)
3
2
1
0
1
2
3
4
5
6
Time (h)

At the end of one hour the maximum incorporation level was reached and the incorporation level of the azadirachtin-injected locusts was 27% that of the controls. Consequently azadirachtin strongly inhibited the incorporation of amino acids into the ovary.

5.3.4.5. Incorporation of ^{3}H-glycine into the proteins of mid gut

The level of incorporation into the mid gut protein is same as in the ovary and the results of the incorporation of ^{3}H-glycine into the mid gut is shown in Fig. 5.8. The equilibrium level of incorporation can be seen 1 hour after injection of radiolabelled glycine. Azadirachtin inhibited the incorporation of ^{3}H-glycine into the mid gut proteins compared to the control locusts and at the end of the 1 hour period the incorporation level of azadirachtin-treated locust was 40% that of the controls.

5.3.5. Incorporation of ^{35}S-cysteine into the brain and corpus cardiacum

The incorporation level of ^{35}S-cysteine into the proteins of brain and corpus cardiacum 4 hours after injection is presented in Table-5.1. The 70% methanol-soluble fraction contained peptides and the free amino acids. The corpus cardiacum showed a higher level of incorporation than the brain. The incorporation patterns of ^{35}S-cysteine into the brain and the corpus cardiacum reveal striking differences between the azadirachtin-injected locusts and the control locusts. In the brain and the corpus cardiacum the total radioactivity (both the methanol soluble fraction and the insoluble fraction) was higher in the control locusts than in the azadirachtin-treated locusts. The methanol-soluble fractions of the brain and the corpus cardiacum in azadirachtin-treated locusts were inhibited the incorporation

level by 55%. This demonstrates that the incorporation was rather poor and slow in the azadirachtin-treated locusts

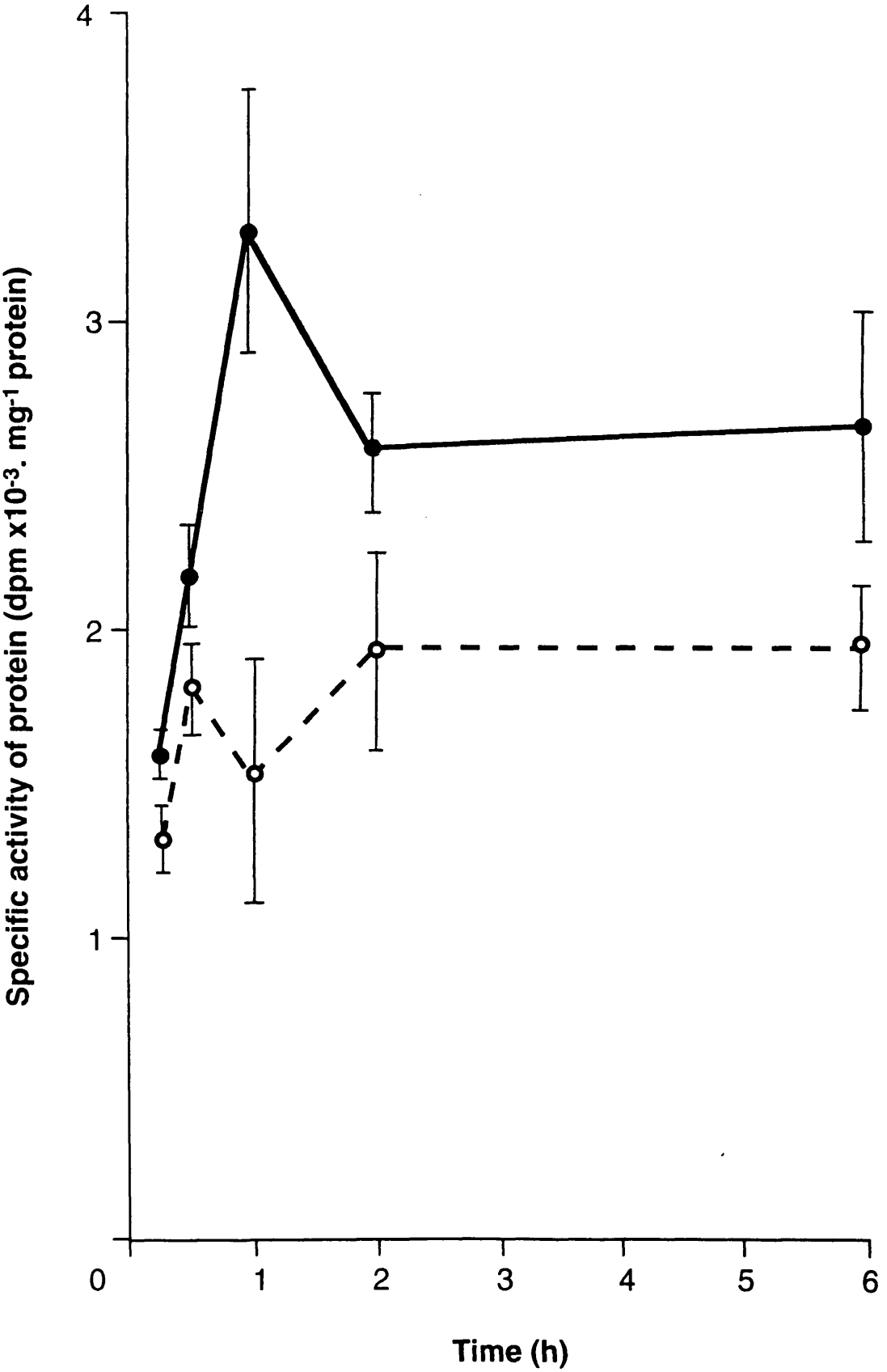
Table-5.1 Incorporation of ^{35}S-cysteine into the corpus cardiacum and the brain.

Tissue	Specific activity (dpm. mg^{-1} of protein)	
	70% methanol-soluble material	70% methanol-insoluble material
Control		
Brain	1625±131	553±62
Corpus cardiacum	2130±260	1732±92
Azadirachtin treated		
Brain	755±56	152±31
Corpus cardiacum	928±91	631±52

A dose of ^{35}S-cysteine (2μCi) was injected into the haemolymph of the female insects, 7 days after the final ecdysis. After 4 hours, the tissue was dissected out and homogenized in 70% aqueous methanol to extract the soluble radiolabelled material and precipitate the protein, which was then redissolved in S.D.S. before estimation. Specific activity of 70% methanol-soluble material was estimated with respect to the precipitated protein. The results are the means (±SD) of 5 insects and the effects of azadirachtin were significant at below p=0.01.

Fig.5.8. The incorporation of ^{3}H-glycine into the mid gut of the locusts

The time course incorporation of ^{3}H-glycine into the mid gut protein of the locust after injection of 2μCi/g body weight into the haemolymph of 7 days old (after the final ecdysis) female *S. gregaria.* was measured. The experimental locusts (○) were injected with 3μg azadirachtin/g 12 hours before injection of radiolabelled amino acid and the control locusts (●) received same amount of 10% ethanol water. The values represent the mean (±SD) of 4 locusts. The effects of azadirachtin were significantly different from the control experiment.


Specific activity of protein (dpm x10-3. mg-1 protein)
4
3
2
1
0
1
2
3
4
5
6
Time (h)

5.3.6. Effect of extracts of corpus cardiacum and azadirachtin on the rate of incorporation of ^{3}H-glycine into specific tissues

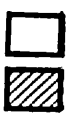
Fig.5.9 shows the effect of neurohormones and azadirachtin on the level of incorporation of ^{3}H-glycine 2 hours after injection. The tissues taken for the analysis were the fat body, the haemolymph, the ovary, and the mid gut. Both the fat body and the ovary showed similar pattern of results whereas the midgut showed different response. In the preliminary experiment, the control locusts showed higher level of incorporation than in the azadirachtin treated locust as described above (Fig.5.9.a). In order to determine the neurohormonal effect on the level of incorporation, the locusts were ligatured 12 hours before injection of radiolabelled glycine, subsequently the effect of azadirachtin was also examined by injecting it into the haemolymph of the ligatured locust (Fig.5.9.b). In both groups the levels of incorporation into the fat body, the haemolymph and the ovary were very similar to each other and the uptake of glycine into the protein of these locusts was dramatically reduced to below 50% that of the control. The midgut showed high levels of incorporation in both ligatured locusts and azadirachtin-treated locusts but this level was lower than in the control locusts.

To establish the effect of an extract of corpus cardiacum on the uptake of glycine into the proteins more precisely, the extract of corpus cardiacum was injected into both control and azadirachtin-treated ligatured locusts 3 hours before injection of radiolabelled glycine (Fig.5.9.c). The results show that the neurohormonal extract increased the uptake of glycine into proteins of the tissues in ligatured insects but the azadirachtin-treated locusts did not show the stimulatory effect of the corpus cardiacum extract. This was true for all tissues examined.

Fig.5.9. Effect of corpus cardiacum extract and azadirachtin on incorporation of ^{3}H-glycine into protein of specific tissues.

2 hours after injection of 2μCi/g body weight into the haemolymph of female *S. gregaria* 7 days after the final ecdysis, the incorporation of ^{3}H-glycine into the specific tissues were measured. The experimental locusts were injected with 3μg azadirachtin/g 12 hours before injection of radiolabelled amino acid and the control locusts received same amount of 10% ethanol water. (a) control experiment; (b) locusts were head ligatured 12 hours before injection of ^{3}H-glycine; (c) locusts were head ligatured 12 hours before injection of ^{3}H-glycine and then the extract of corpus cardiacum was injected into the haemolymph 3 hours before injection of ladiolabelled amino acid. The values represent the mean (±SD) of 4 locusts.

1. Fat body
2. Haemolymph
3. Ovary
4. Mid gut



Control locust

Azadirachtin-treated locust

* Significantly different from the appropriate control experiment

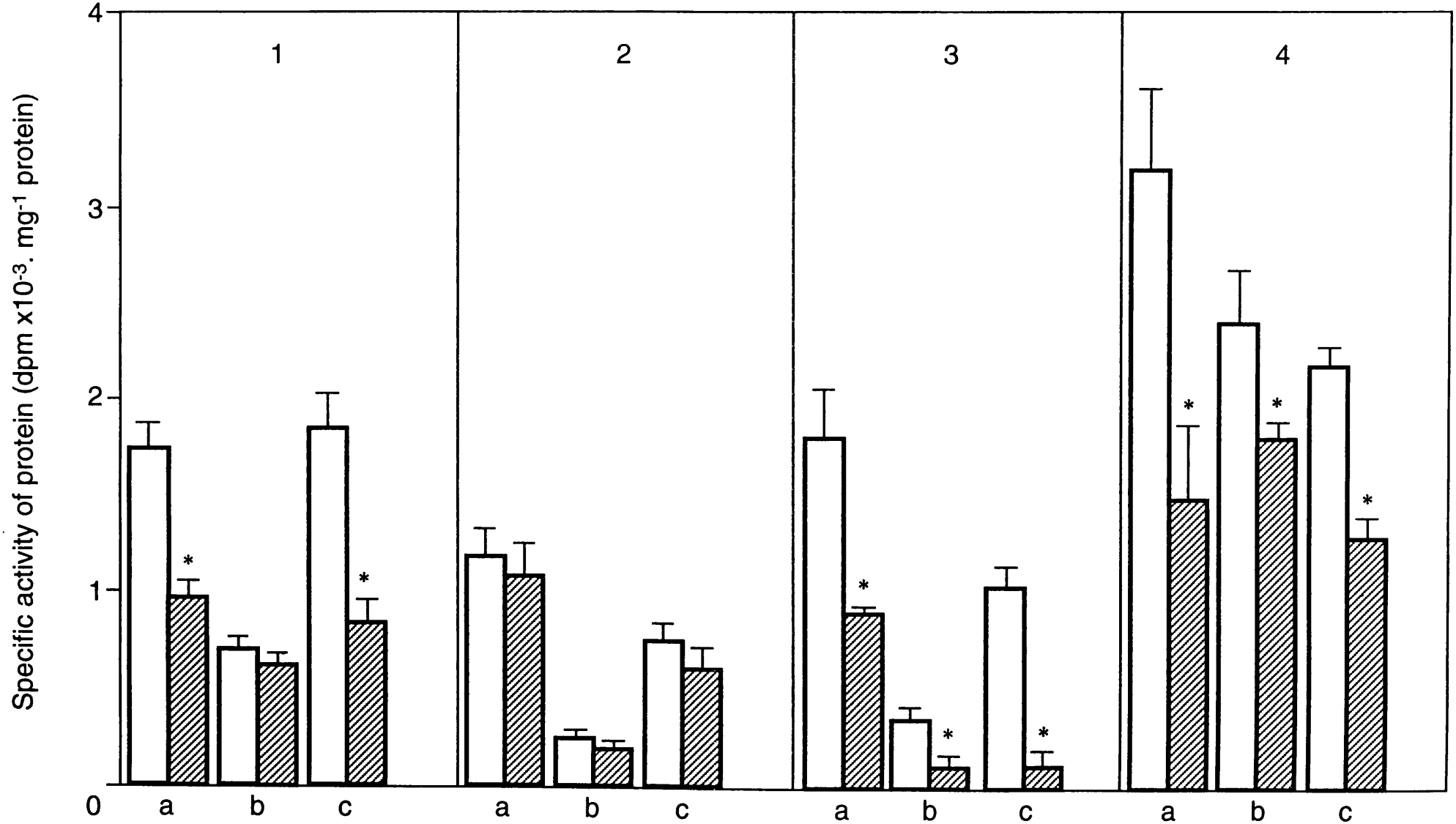

Specific activity of protein (dpm x10-3. mg-1 protein)
4
3
2
1
0
1
2
3
4
a
b
c
*

Fig. 5.10 shows the clearance of ^{3}H-glycine from the haemolymph of ligatured control locusts and ligatured azadirachtin-treated locusts compared with results of the unligatured insects. The results show that the rate of removal of labelling in the haemolymph is not affected by ligation or azadirachtin.

5.3.7. Experiments *in vitro*

5.3.7.1. Incorporation of ^{3}H-glycine into the protein of isolated tissues

In Table 5.2 are shown the results of the preliminary experiment to determine the most appropriate medium for the experiments performed *in vitro*. One medium was the simple insect saline, without added amino acids, while the other was a complete insect cell culture medium, with a full range of amino acids. In both cases the uptake reached a maximum by the end of two hours of incubation. By this time, the incorporation of radio-label into TCA precipitable material was four times greater in the tissue incubated in the simple medium. Consequently, all further experimentation using ^{3}H-glycine was done in the simple insect saline, over a 2 hour time period.

Table 5.3 shows the effects of corpus cardiacum extracts and azadirachtin both separately and together, on the incorporation of labelled glycine into proteins of the fat body, the ovary and the midgut. The effect of the neurohormonal extracts was to double the incorporation of label in the fat body, the ovary and to increase it by 60% in the gut. These results were statistically highly significant.

Table-5.2: Effect of the presence of amino acids in the medium on incorporation of ^{3}H-glycine into the fat body protein of the locusts *in vitro*.

Time of incubation (hours)	Specific activity (dpm. mg^{-1} of protein)	
	Insect cell culture medium	Insect saline
1	840	4631
2	2026	8850
4	2036	7931

A dose of ^{3}H-glycine (2μCi) was added to the incubation medium. The cell culture medium contained a full range of haemolymph amino acids which were absent from the saline. After different times of incubation, the fat body was homogenized in 5% TCA to precipitate the protein, which was then washed twice with 5% TCA to remove the unincorporated ^{35}S. The results are the average of duplicate incubations and did not different from each other by > 8%.

The addition of azadirachtin to the incubations had the effect of eliminating completely the effect of the crude hormonal extracts. In the case of the fat body and the ovary, although azadirachtin did reduce the incorporation to a level below that of the control, the reductions were not statistically significant. But in the case of the gut, azadirachtin alone, as well as in the presence of the tissue extract, reduced the incorporation of label to an average of 50% of that of the control value.

5.3.7.2. Heat lability of crude corpus cardium extract

The simple experiment to determine the heat-lability of the extracts of corpus cardiacum showed that a 10 min boiling period eliminated the stimulatory effect on the protein metabolism, suggesting that the active components are liable to heat-inactivation (Table 5.4).

Table-5.3: Effect of the corpus cardiacum extract on the inhibition due to added azadirachtin on the level of incorporation of ^{3}H-glycine into specific tissues of the locusts *in vitro*.

Tissue	Specific activity (dpm. mg^{-1} of protein)			
	Conditions			
	1	2	3	4
Fat body	8850±721a	17550±1032b	6202±658a	7673±580a
Ovary	8919±943a	19096±565b	7546±718a	8431±467a
Mid gut	5432±416a	8597±717b	2212±204c	3367±216c

The incorporation into 5% TCA-precipitable material of the fat body, the ovary, and the mid gut of the locusts was measured under the following different conditions 2 hours after adding ^{3}H-glycine into the medium:(1) control; (2) preincubated with the extract of one corpus cardiacum for 30min; (3) preincubated with both corpus cardiacum extract and azadirachtin for 30min; (4) preincubated with azadirachtin only for 30min. The results are the means (± SD) of 4 insects. The letters indicate statistically significant differences for one tissue, ie, those means with the same letters do not differ significantly. Those with different letters are significantly different at $p<0.01$.

Fig.5.10 Effect of head-ligaturing in the clearance of ^{3}H-glycine from the haemolymph of the locusts.

A dose of 2μCi of ^{3}H-glycine was injected into the haemolymph of the locust at zero time and samples taken as described. Control (▼); locusts head-ligatured 12 hours before injection of ^{3}H-glycine (■); head ligatured as above and injected with the extract of corpus cardiacum 3 hours before injection of ladiolabelled amino acid (●).The points are the mean of 4 locusts, and the vertical bars indicate the standard deviation. The upper curve indicates the semilogarithmic representations of the characteristic of the uptake of ^{3}H-glycine from the locust haemolymph.

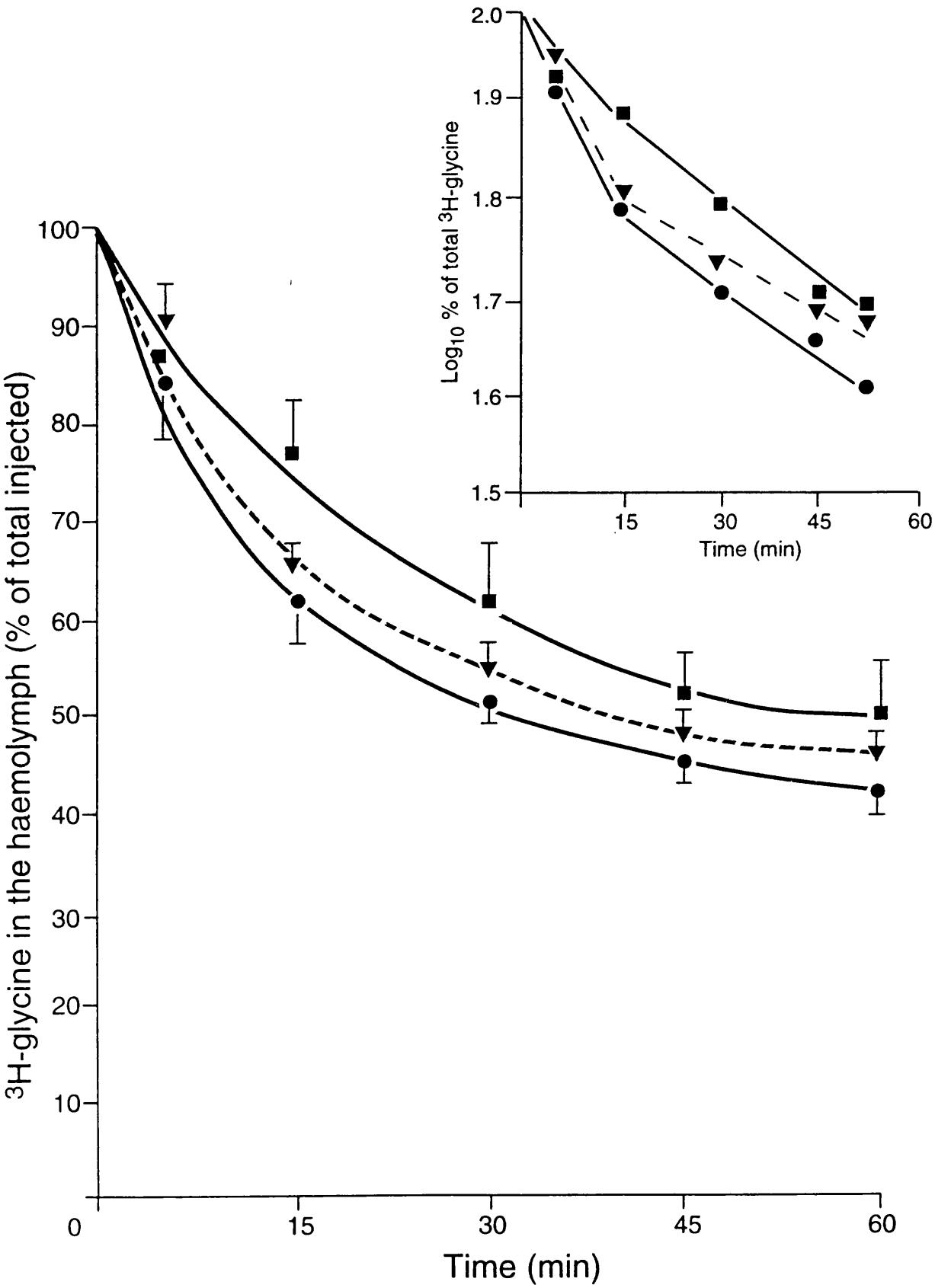

2.0
1.9
1.8
1.7
1.6
1.5
Log10 % of total 3H-glycine
15
30
45
60
Time (min)
100
90
80
70
60
50
40
30
20
10
0
3H-glycine in the haemolymph (% of total injected)
15
30
45
60
Time (min)

Table-5.4: Investigation of the effects of boiled extracts of corpus cardiacum on the incorporation of ^{3}H-glycine into the fat body of the locust *in vitro*.

The experiment	Specific activity (dpm. mg^{-1} of protein)
Control	8859
Unboiled extract	17550
5min boiled extract	15391
10min boiled extract	9110

The fat body was incubated with ^{3}H-glycine and the corpus cardiacum extract and after 2 hours incorporation of the radiolabelled into TCA-precitable material was measured and compared with control experiment. The results are the means of duplicate experiments which did not different from each other by > 10%.

5.3.8. Incorporation of ^{35}S-cysteine into the brain and corpus cardiacum

The incorporation of ^{35}S-cysteine into the corpus cardiacum and the brain were measured 4 hours after addition of the radiolabelled amino acid to the medium. The incorporation patterns of radiolabel into the protein of 7 day old females revealed striking differences between the azadirachtin-treated tissues and the controls. The 70% methanol-soluble material of both control and azadirachtin-treated tissues were separated into two clearly resolved fractions on the gel filtration column. All the high molecular weight compounds were eluted with vitamin B_{12} and this fraction contained the compounds of molecular weight higher

than 1300. The low molecular weight fraction, eluted with the lysine derivative, contained the compounds of molecular weight less than 1300, including all free amino acids and reduced and derivatised glutathione.

Table-5.5 Effect of azadirachtin on incorporation of ^{35}S-cysteine into the brain and corpus cardiacum *in vitro*

	Specific activity (dpm. mg^{-1} of protein x 10^{-3})		
	70% methanol-soluble material		70% methanol-insoluble material
Tissue	Low molecular weight fraction	High molecular weight fraction	Protein
Control			
Brain	223.1±4.4	91.0±9.0	15.1±1.8
Corpus cardiacum	314.8±2.2	141.8±3.1	33.1±3.8
Azadirachtin-treated			
Brain	80.5±3.7	38.1±4.1	8.9±1.0
Corpus cardiacum	84.3±9.1	53.9±5.8	18.4±1.1

A batch of 12 brains and corpora cardiaca were incubated separately, with 2 μCi of ^{35}S-cysteine for 4 hours and homogenised in 70% aqueous methanol to extract the peptides and the other soluble materials. The precipitated proteins were redissolved in S.D.S before estimation. The methanol-soluble fraction was freeze dried and redissolved in 0.1 ml of methanol and passed through the G-10 sephadex column to resolve the labelled material into two fractions: low molecular weight fraction (M_r<1300) and high molecular weight fraction (M_r>1300). The radioactivity of each fraction was measured and the specific activity of the 70% methanol-soluble material was estimated with respect to the precipitated protein. The results are means (±SD) of 4 samples and the effect of azadirachtin was significant at below p=0.05.

Although the incorporation of radiolabelled amino acid into the protein of the corpus cardiacum was higher than that of the brain, both the brain and the

corpus cardiacum showed 70% of the total radiolabel incorporated into the protein was in the low molecular fraction, 27% of the total was detected in the high molecular weigh fraction and only 3% was found in the precipitable protein (Table-5.5). In general, the uptake of the radiolabel into the protein of the corpus cardiacum of the azadirachtin-treated locusts was not significantly higher than the brain as control experiment. This confirmed that the incorporation was rather poor in the azadirachtin-treated tissues. Although azadirachtin does not effect to the uptake of radiolabel in the neurosecretory proteins, the total ^{35}S-cysteine retained in the brain and corpus cardiacum was comparatively low in azadirachtin-treated locusts. These results are comparable with the *in vivo* results.

5.3.8.1 Uptake of ^{35}S-cysteine into the brain and the corpus cardiacum

Fig.5.11 shows the rates of clearance of ^{35}S-cysteine from the medium into the brain and the corpus cardiacum during a 6 hour incubation. The results show that the rate of uptake is more rapid into the brain than the corpus cardiacum but that azadirachtin did not affect the rate of uptake. For both tissues the effect of azadirachtin showed an identical curve to the control experiment and therefore only one is shown in the figure.

5.3.9. Metabolism of ^{35}S-cysteine in the brain and corpus cardiacum by TLC

The radioactivity incorporated into the peptides was assessed using TLC. Radioactivity was detected in the bands corresponding to cysteine, cystine, glutathione (oxidised), and the origin which was indicated by addition of carrier quantities of standard of sulphur-containing amino acids, visualised with ninhydrin. Then the plate was divided into 1 cm bands which were scraped off.

Fig.5.11 Direct effect of azadirachtin on uptake of ^{35}S-cysteine into the brains and corpora cardiaca of the locust *in vitro* .

Brains and the corpora cardiaca of 4 locusts were preincubated in the insect saline containing azadirachtin for 30 min before the addition of 0.25μCi of ^{35}S to the medium and samples were taken as described. Control tissues received same amount of 10% ethanol/water. Both the results of control and azadirachtin-treated tissues were identical, therefore only one is shown. The brain (●); The corpus cardiacum (○). The results are the means of duplicates.

Radioactivity remaining in medium
(% of total present)
100
90
80
70
60
50
1
2
Time (h)

There was therefore evidence for ^{35}S-cysteine metabolism in the brain and the corpus cardiacum but this was higher in the brain. In order to estimate the potential ^{35}S incorporated peptides that might interfere with an oxidised glutathione, the extracts were derivatised with NEM. Fig.5.12 and 5.13 show resolution of sulphur-containing amino acids on a cellulose TLC plate after staining with ninhydrin and TLC of the 70% methanol-soluble fraction of the brain and the corpus cardiacum, after reaction with $NaBH_4$ and NEM respectively. The TLC indicated that the activity on the base line was contaminated by ^{35}S labelled oxidised glutathione, but this was solved by reduction with $NaBH_4$. Oxidised and reduced standards of glutathione were included since these are known to incorporate ^{35}S after administration of ^{35}S-cysteine to insects (Cotty et al., 1958).

5.3.10. Effect of azadirachtin on juvenile hormone-induced protein synthesis in the fat body *in vitro*.

Adult females were allatectomized chemically by treatment with ethoxyprecocene 12 hours after the final ecdysis and the effectiveness of the treatment was visualised by lower development of the fat body in the locusts 7 days later. The results showed that after the hormone treatment, the incorporation level of the fat body was 60% higher than the control (Table-5.6). This effect was statistically highly significant. Azadirachtin, however, did not inhibit the stimulatory effect of the hormone.

Fig.5.12. The TLC chromatogram of standard 'S' containing amino acids.

The figure represents a TLC of standard sulphur-containing amino acids, oxidised and reduced forms of glutathione as well as the N-ethyl maleimide (NEM) derivatives of cysteine and glutathione on cellulose plates after double development in butanol: acetic acid: water (11:6:3). After resolution, the chromatogram was stained with ninhydrin.

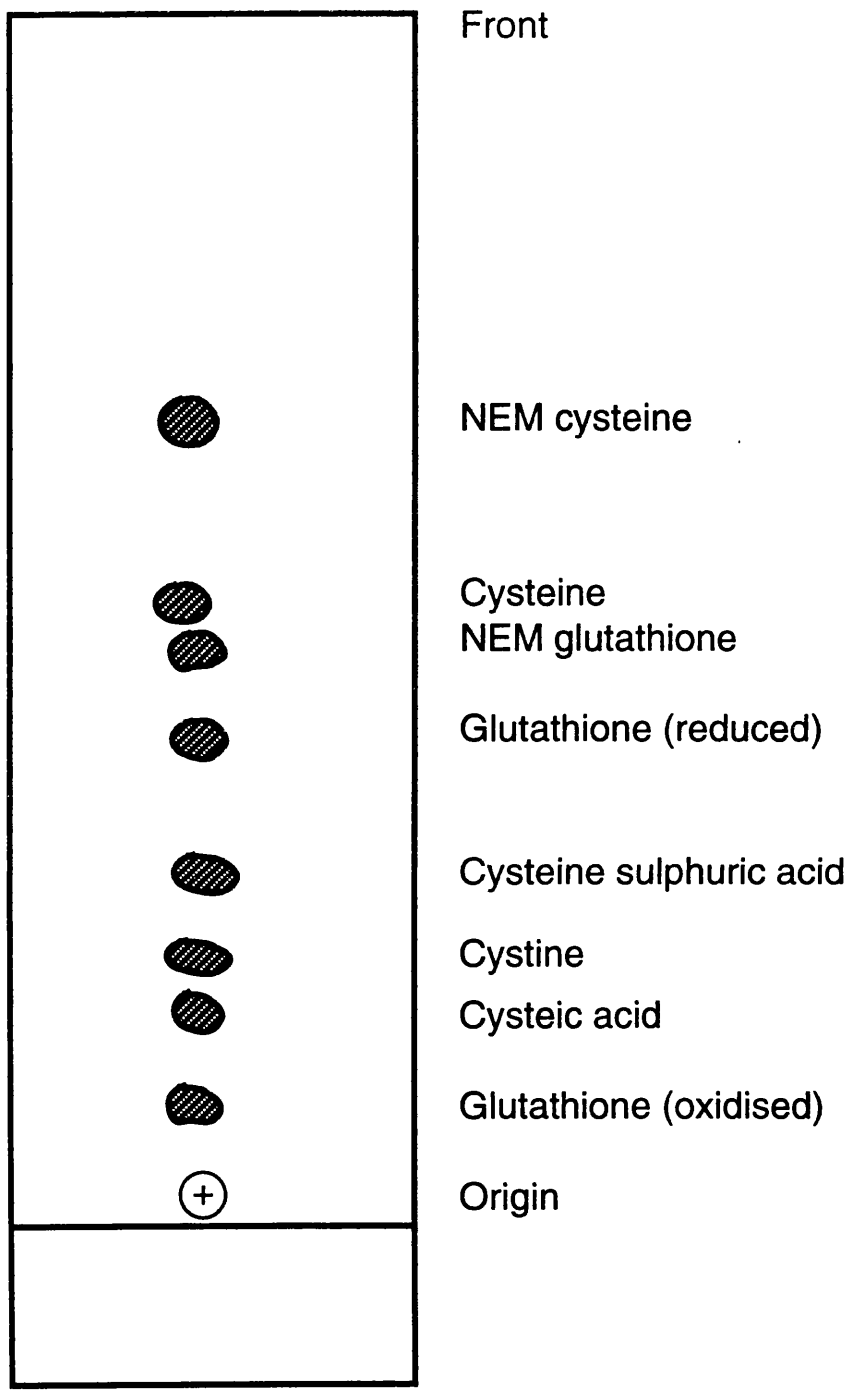

Front
NEM cysteine
Cysteine
NEM glutathione
Glutathione (reduced)
Cysteine sulphuric acid
Cystine
Cysteic acid
Glutathione (oxidised)
Origin

Fig.5.13 Incorporation of radiolabel from ^{35}S cysteine into 70% aqueous methanol-soluble material in the brain and corpus cardiacum *in vitro*.

The figure represents the results of chromatography on cellulose of the 70% methanol-soluble extract of the brain (a) and corpus cardiacum (b) after incubation *in vitro* with ^{35}S cysteine and treatment of the extract with $NaBH_4$ and NEM. The spots represent the location of (1) NEM cysteine; (2) NEM glutathione. After double development in butanol:acetic acid: water (11:6:3) (v:v:v), 1cm strips of the cellulose were scraped into scintillation vials, and the radio-activity counted.

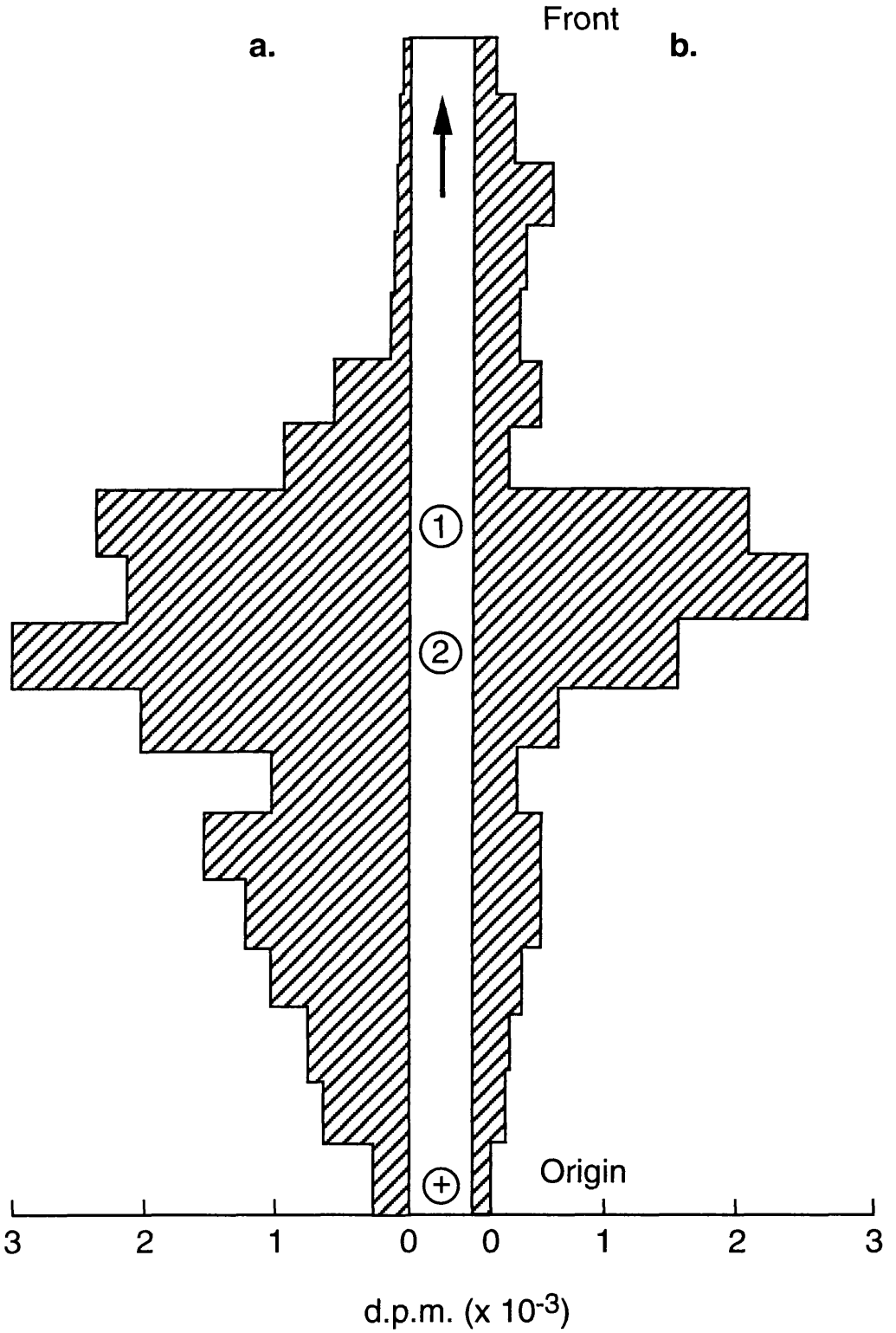

Front
a.
b.
1
2
+
Origin
3
2
1
0
0
1
2
3
d.p.m. (x 10-3)

Table-5.6 Effect of azadirachtin on juvenile hormone-induced protein synthesis in the fat body *in vitro*

Additions to medium	Specific activity dpm. mg^{-1} of protein
Control	5580±631a
Juvenile hormone	8930±963b
Juvenile hormone and azadirachtin	8152±526b

The female locusts were allatectomized chemically by treatment with ethoxyprecocene and the fat body was dissected and incubated with 60 µg of the juvenile hormone analogue, Methoprene for 24 hours in the cell culture medium. Then the rate of incorporation of ^{3}H-glycine was measured. The fat body was preincubated in the insect saline with JH and azadirachtin before being incubated with ^{3}H-glycine to check the effect of azadirachtin. The results are means (±SD) of 4 samples and different letters indicate statistically significant differences at $p \leq 0.01$.

Discussion

The experiments reported in Chapter 5 were performed to examine the effects of azadirachtin on protein and peptide synthesis in various tissues of the adult female locust by following the incorporation of two radiolabelled amino acids into proteins and peptides. The age of the locusts used was 7 days after the final ecdysis because these represent the peak of protein synthesis in maturing females. The two amino acids glycine and cysteine were selected as appropriate for following protein synthesis in the different tissues based on previous work (Mordue et al., 1973; Hill, 1962). These two amino acids showed similar patterns of uptake (see section 5.2.2.6), although ^{35}S-cysteine showed higher specific activity per mg of protein than ^{3}H-glycine.

Cysteine adsorbed into tissues and their proteins due to the possible formation of dithiol artefacts. Because of its sulphurhydral group, cysteine is unstable in aqueous medium, and easily oxidises to form the dimer cystine, or forms dithiol links with cellular proteins containing cyteine residues (Newton et al., 1992) This will tend to give artificially high rates of incorporation. It was for this reason that the studies looking at the incorporation into "neuropeptides" were done with extracts which had been reduced with NaBH4 and then derivatised.

It was further clear that the two methods of protein precipitation employed here were intended to achieve only a crude separation of polypeptide fractions, and they were used for different purposes. 5% TCA will precipitate polypeptide material with molecular weights over 5000. Thus the pellet is likely to contain most labelled amino acids which have been incorporated into a polymeric form. These experiments were mainly concerned with the precipitated material, i.e. the incorporation into "proteins." On the other hand, various proportions of aqueous methanol were employed to ensure that the possible neuropeptides remained in solution. 70% aqueous methanol was used here following the work of Girardie et al (1985) and Subrahmanyum et al (1989) in extracting neuropetide material from the neurohaemal organs of locusts. These workers found that some polypeptides of very high molecular weight (M_r40,000-120,000) were soluble in the methanol. Thus the methanol method cannot achieve a separation between high molecular weight "proteins" and low molecular weight "peptides". It was for this reason that the methanol-soluble fraction was further resolved by exclusion chromatography.

One of the problems in trying to determine the primary actions of azadirachtin is that it has such a general toxicity that the picture is rapidly complicated by the appearance of secondary effects. For instance, the secondary antifeedant effect of azadirachtin causes the insect to cease feeding, and thus produces an effect of starvation. Highnam et al (1974) showed that the uptake

from the haemolymph of an injected radiolabelled amino acid was much slower in locusts which had been starved for 5 days than in normally-fed controls. This may partly explain the results of Subrahmanyam et al (1989) who found that the uptake of ^{35}S-cysteine from the haemolymph of locusts was also very slow in those insects which had been treated 7 days before with azadirachtin (3μg/g). These effects were avoided in the work reported here by using short time-courses for the experiments, and by ensuring that control insects were starved to mimic the antifeedant effects of azadirachtin . Uptake of labelled amino acids was the same in 12 hours starved and azadirachtin-treated locusts as in fed control, eliminating this aspect as one of the possible reasons for an effect on protein synthesis. In both *in vivo* and *in vitro* experiments, no evidence was obtained for a direct effect of azadirachtin on the uptake of either amino acid into any of the tissues examined.

Measurements of haemolymph levels of radiolabel show that approximately 40% of the radioactivity was lost from the haemolymph 30min after the injection. The rates of uptake of both amino acids are exponential, but do not follow a simple pattern. At least two separate exponentials with different rate constants may be distinguished. Within the first 30 minutes the uptake is very fast, suggesting most of the radiolabel can be taken up into the widely distributed fat body where much protein metabolism takes place. These results are consistent with the results published by Mordue et al (1973) and Subrahmanyam et al (1989) for untreated locusts.

When a physiologically effective dose of azadirachtin (3μg azadirachtin/g body weight) was injected, it induced an inhibitory effect on the incorporation of both radiolabelled amino acids into the protein of the whole locusts suggesting a general effect.

In untreated locusts, the sequence of incorporation of labelled glycine into the proteins of the fat body, haemolymph and ovary differs slightly from that

previously reported by Hill, 1965. The previous authors found a clear progression of label first into fat body then haemolymh, and finally ovary, consistent with the idea that the source of the bulk of the ovarian proteins is the fat body. The results here suggest a much higher and earlier incorporation into the ovary, compared to the haemolymph, suggesting that the ovary is more capable of synthesising its own proteins than was thought before. This is confirmed by the fact that azadirachtin acts directly upon the neuropeptide-stimulated protein synthesis in the ovary, but has no influence on the effects of JH which has been shown to induce very specifically vitellogenin.

The profound effect of azadirachtin on reducing the incorporation of label into ovarian protein is likely to be due mainly to a direct effect on that tissue. This would help to explain the fact that the clear-cut reduction of protein synthesis by azadirachtin shown by fat body and ovary is not apparent in haemolymph. The fact that azadirachtin does not apparently interfere with the actions of JH on fat body means that there would be little effect on the appearance in the haemolymph of vitellogenin.

Although it is clear that the neuroendocrine system is involved in the control of protein turnover in the fat body of the female desert locust, the effect of the neurosecretion system is complicated. Following elimination of neurohormone release by head ligation, the level of incorporation of both the azadirachtin-treated and the control locusts was significantly reduced, but only in the control head-ligatured female locusts could the incorporation level be restored to normal (Fig.5.9) by injection of an extract of corpus cardiacum 3 hours before injection of the radiolabelled amino acid. This suggests that azadirachtin is having a specific effect on the actions of corpus cardiacum extract. These results are comparable with the *in vitro* results which demonstrate that the tissues incubated with a corpus cardiacum extract showed significantly higher incorporation rates in the control

locusts but not for azadirachtin-treated locust tissues. It is probable that the low level of incorporation following cautery of the cerebral neurosecretory cells is the the result of such a direct effect upon protein synthesis. Either cautery of the cerebral neurosecretory cells or allatectomy in the desert locust results in the inhibition of oocyte growth (Highnam, 1962; Highnam et al., 1963). In the immature female locusts following both these operations, the incorporation rate of ^{14}C-glycine into the fat body protein does not increase, as it does in control animals, but slowly decreases. The decrease of incorporation is more rapid after neurosecretory cell cautery than after allatectomy, and the incorporation rate reaches a lower level (Hill, 1965). In other insects the corpora allata have been implicated in the control of protein synthesis. Allatectomy of *Rhodnius* results in a decrease in the incorporation of leucine into the protein of the fat body (Vanderberg, 1963)

The direct effect of azadirachtin on the fat body, the ovary and the haemolymph are consistent with prevention of stimulatory action of neurohormones on protein synthesis but the mid gut shows a different type of direct effect. The level of incorporation of amino acids into the gut protein of the head-ligatured control locusts is higher than the ligatured azadirachtin-treated locusts suggesting an additive effect (Fig.5.9). The incorporation level of both these group is lower than in control insects. Azadirachtin apparently has a direct effect on protein synthesis of the mid gut which is independent of neurohormones. The direct effect of azadirachtin upon gut muscle contraction both *in vivo* and *in vitro* has been recorded (Mordue et al., 1985) and it reduces the activity of most of the digestive enzymes which are secreted by the mid gut (Timmins et al., 1992). The inhibition of gut contraction by azadirachtin has a marked effect on passage through the gut. This results not only in lower faecal production but in lower rate of absorption of food. Studies of mid gut tissue show that the epithelial cells are much disrupted by the action of azadirachtin (Cottee, 1984; Mordue, 1985). There is also suppressed

level of feeding, because feeding is not initiated in the locust until the foregut and hindgut are relatively empty (Bernays et al., 1973; Simpson, 1983). Although the injection of azadirachtin into the haemolymph bypasses the azadirachtin-sensitive chemo-receptors on the mouthparts (Blaney et al., 1980), an antifeedant effect is still produced by its effect on the passage of food through the gut. The effect of neurohormones on the mid gut of the other insects has also been studied. It is clear that the neurosecretory system can affect the synthesis of digestive enzymes of the mid gut (Thomsen et al., 1963). As described in the Introduction, there is evidence that azadirachtin inhibits digestive enzyme secretion. In *Manduca sexta*, azadirachtin inhibits the production of trypsin by the enzyme-secreting cells of the mid gut wall and in *Spodoptera litura* azadirachtin significantly affects the digestive enzymes such as protease, amylase and invertase. Therefore, the low level of incorporation of amino acid into the gut proteins in the azadirachtin-treated locust would be due to the secondary antifeedant effect of azadirachtin. Thus a direct effect of azadirachtin on gut protein synthesis can account for some part of secondary antifeedant effect.

The turnover of ^{35}S-cysteine in the brain and the corpus cardiacum was compared between azadirachtin-injected and control locusts starved for 12 hours. The rate of ^{35}S-cysteine incorporation demonstrates that the azadirachtin-treated locusts have a very low turnover of neurosecretory protein in the brain and the corpus cardiacum. In control locusts, in which the oocyte development is rapid, the transport of ^{35}S-labelled protein from the brain to the corpus cardiacum and its subsequent release are at significantly higher levels. The neurosecretory activity of the female *S. gregaria* was studied under various physiological conditions (Mordue et al., 1973) and, in relation to feeding (Highnam et al., 1974), by following the incorporation of ^{35}S-cysteine into the products of neurosecretory cells. These studies demonstrated that in maturing females (10 days old), synthesis and release

of neurosecretory material is fast compared to immature females. Mature females have a much less active system than maturing or immature females. Incorporation of ^{35}S-cysteine into the median neurosecretory cells of starved females was low and transport and release of neurosecretory material minimal. The effects of azadirachtin are not comparable to starvation-induced effects on neurosecretory turnover, because the incorporation of ^{35}S-cysteine into the brain and the corpus cardiacum of azadirachtin-injected locusts was significantly lower than in starved insects.

In the azadirachtin-treated locusts, the transport of labelled protein from the brain to the corpus cardiacum and its release are at a very low level, though not completely inhibited. Hence, inhibition or disturbance of ovarian development in azadirachtin-treated females is mainly due to the changes induced in the endocrine system by the poor turnover of neurosecretory proteins. Synthesis and release of neurosecretory material are very rapid in maturing females (Highnam et al., 1974). Study of this process by labelling the protein clearly differentiates the transport and the release phases. As the ^{35}S-cysteine concentration of the haemolymph rapidly falls, it becomes limiting as early as 2 hours after injection due to adsorption, interchange of the label with other tissues and excretion of the amino acid (Mordue et al., 1973).

The incorporation level of ^{35}S-cysteine in the brain and the corpus cardiacum was measured *in vitro*. These results were very similar to the *in vivo* results. The metabolism of ^{35}S-cysteine was studied using TLC and exclusion chromatography of 70% methanol soluble fractions of the brains and corpora cardiaca. The TLC results indicated that most (60%) of radiolabel is due to oxidised glutathione and this obscured incorporation into other peptides which remained on the origin of the TLC plate. To solve this problem, oxidised and protein-bound thiols were converted to their reduced counterparts by the use of $NaBH_4$, followed

by derivatization with NEM. Previous workers have failed to confirm that most of the incorporation of cysteine into "peptide" was glutathione (e.g.Subrahmanyam et al., 1989).

In order to modulate true incorporation into high molecular weight peptides, the reduced 70% methanol fractions of the brains and the corpora cardiaca were further analysed by exclusion chromatography. Glutathione was eluted with the low molecular weight fraction which contained all the other free amino acids and small peptides with molecular weight less than 1300. According to the results published by Girardie et al. the high molecular weights fraction contains all the neurosecretory proteins. The molecular weight of these proteins is approximately 54,000 - 123,000. In agreement with these investigations, it was observed that the rate of incorporation is higher in the corpus cardiacum than in the brain. However, the incorporation of the label in the high molecular weight fraction is reduced in the azadirachtin-treated locusts. Thus, in the treated tissues, the appearance of labelled neurosecretory proteins is quantitatively reduced 4 hours after administration of ^{35}S-cysteine. Subrahmanyam et al. (1989) was also working with 70% methanol soluble material of corpus cardiacum and found that although there was a quantitative reduction of incorporation of ^{35}S-cysteine into peptide material of the azadirachtin-treated locusts, the same six fractions were present in the extracts of corpus cardiacum of both control and azadirachtin-treated locusts. More than 75% inhibition due to azadirachtin can be seen in the corpus cardiacum but the brain shows only 65% inhibition. Of the various endocrine centres investigated, azadirachtin is highly concentrated in the corpus cardiacum relative to the brain (Rembold et al., 1989) suggesting that the lower level of synthesis of neurosecretory hormones and the accumulation in the corpus cardiacum are due to this high concentration of azadirachtin in the corpus cardiacum.

In summary, the results reported here suggest that azadirachtin interferes with polypeptide biosynthesis in many tissues of the locust. Probably the most important effects are those on the formation of the neuropeptides of brain and corpus cardiacum, which control so much of the metabolism and biology of the insects. The results suggest that neuropeptides stimulate the biosynthesis of proteins in several tissues including fat body and ovary, which would thus indirectly reduce female fecundity. It has yet to be proved however, that azadirachtin lowers the titre of any identified peptide neurohormone in the haemolymph.

In addition to these effects, there is evidence that azadirachtin has also an effect of preventing the stimulatory effects of some neurohormones on protein biosynthesis on some tissues. The fact that azadirachtin dose not appear to interfere with the effects of JH on fat body suggests that here is a system which may allow studies to find the mode of action of the terpenoid in this tissue.

CHAPTER 6

FINAL DISCUSSION

Research into the insecticidal effects of azadirachtin, a limonoid from the Indian neem tree, *Azadirachta indica* , and its close analogues, has been ongoing for some 30 years. Many biological effects of azadirachtin are now well documented, although its biochemical effects at the cellular level are still unknown. The effects of the tetranortriterpenoid can be divided into two broad categories: the "primary" antifeedant and repellent effects, and toxic effects including the "secondary" antifeedant effects, and those on growth and development, which are slower to become apparent. Both of these types of effect are presumably dependent upon the presence in insects of specific, high-affinity receptors for the tetranortriterpenoids. Those mediating the primary antifeedancy must be external, on the antennae or the mouthparts, while the other effects must be due to internal receptors, interfering with cellular events, maybe through some second messenger system. The work presented in this thesis deals only with the latter category.

Until some specific receptor is isolated, the existence of such an entity will not be definitely proved, but the results presented here are consistent with the presence of at least a specific high-affinity uptake mechanism for the terpenoids in the locust tissues. The fact that the pharmacology of inhibition of uptake by analogues seems to echo closely the bioassay based on the development of *Epilachna varivestrsi* suggests that there are specific binding/uptake sites which are related in some way to biological effects, and that they may span species boundaries. This gives some reassurance that the results obtained only with the desert locust may have a wider validity. The lack of effect of salannin on both uptake and general development is also a strong argument in favour of the receptor hypothesis. Further support is given by the fact that azadirachtin and its

analogues affect the metabolism of cultured cells derived from the ovary of *Spodoptera frugiperda*, but have no effect on cultured mammalian cells.

The results presented here, and those of other workers, indicate that azadirachtin and dihydroazadirachtin bind very firmly once they have entered the insect cells. It is not clear if this binding is at the level of the plasma or intracellular membranes, of some cytoplasmic or even nuclear receptor. The failure to find any effect of the terpenoids on Malpighian tubule function argues against any effect on mechanisms mediated by c-AMP, but does not rule out other second messenger systems. If, as seems likely, one major effect of the azadirachtin is in preventing the transcription of some proteins, as a result of neurohormone action, it may be that the final binding site may be some cytoplasmic or nuclear protein or transcription factor.

The main achievement of the work presented here is that it confirms and unites previous work which suggests that one of the main effects of azadirachtin is to interfere with polypeptide biosythesis in various insect tissues. Most important from the point of view of explaining the well-known effects on many aspects of insect development is that there is clear evidence that a major effect is the reduction of incorporation of radio-labelled sulphur into the proteins and peptides of the brain and corpus cardiacum. The fact that the peptide neurohormones are rich in cysteine residues, suggests that this reflects a sharp reduction in the synthesis of these hormones. Many of the developmental processes are ultimately controlled by polypeptide neurohormones originating from the pars intercerebralis, and released via the corpus cardiacum. No analysis of individual brain proteins was done here, but the work of Subrahmanyam et al (1989) showed a reduced production of all the main sulphur-containing

proteins soluble in 70% methanol, which would be consistent with the results presented here. It must be said, however, that evidence for the reduced production of neurohormones is not wholly consistent with those results which showed an accumulation of fuschin-staining material in the neurosecretory cells of the brain, suggesting that the effect of the terpenoid is on release or transport rather than on synthesis. This point requires further investigation.

It is possible that many aspects of "loss of fitness" can be attributed to reduction of peptide neurohormone availability, with the resulting loss of metabolic control.

One facet of "loss of fitness" is the "secondary antifeedant" influence of the terpenoids. Although this ill-defined effect has a complex aetiology, at least some of it may be attributed to reduced production of digestive proteases resulting from the direct effect of azadirachtin on protein biosynthesis in the mid-gut.

It seems clear that although there is a very general effect of azadirachtin on protein biosynthesis, its mode of action varies from tissue to tissue, and is selective even within one tissue. Thus while the effect on brain and gut appear to be general, the effect on fat body and ovary is only to negate the stimulatory effects of neurohormonal extracts. It is clear from the lack of effect on the protein biosynthsis induced by juvenile hormone, and from the results of Annadurai and Rembold (1993), that only some proteins are affected. In the light of these differences, it is difficult to propose a single common mechanism underlying an apparently general effect on proteins synthesis.

The work presented here adds some further empirical information to practical questions concerning the possible use of azadirachtin as a

pesticide. Clearly many locust tissues take up the compound and its analogues with great avidity, but have little capacity to detoxify it. It is retained in the tissues for a surprisingly long time. The dihydroderivative is equally or even more effective in its toxic action than the unsaturated azadirachtin. This may be of practical significance, as the saturation of the double bond is said to make the dihydroderivative less susceptible to destruction in the field by UV light.

On the negative side, the high polarity of the terpenoids means that they penetrate the cuticle only poorly, in comparison with many of the synthetic neurotoxic pesticides. This suggests that the optimal mode of application may be indirect, as a systemic pesticide, rather than by direct spraying on to the insects.

The structural complexity of azadirachtin and its analogues precludes its syntheses for pesticidal use. However, it is clear from this and other studies that some of the effects shown by the whole molecule can be mimicked by partial structures such as the decalin and furanoid ring systems. As these can be easily synthesised on a large scale, there is hope of a synthetic industry based on the production of crucial parts of the whole tetranortriterpenoid molecule.

The present results, although confirming that a major effect of azadirachtin appears to be on protein synthesis, leaves many questions unanswered, not least among them the fact that different tissues seem to respond in different ways to the terpenoid. Can the general effects on protein synthesis in the gut and brain, operate through the same mechanism as the indirect effects on fat body and ovary?

These questions may best be approached by the use of an appropriate target tissue, which has been shown to respond to azadirachtin.

The effects found here on ovarian tissue *in vitro* suggest that the line of cultured cells derived from the ovary of *Spodoptera frugiperda*, Sf9, will be an excellent system for the futher study of many of the unanswered questions, in very clearly defined conditions. These *in vitro* studies must however be constantly measured against the yardstick of the true situation *in vivo*.

REFERENCES

Abdul Kareem, A. (1981) Neem as an antifeedant for certain phytophagous insects and bruchil on pulses, in Natural Pesticides from the Neem Tree (*Azadirachta Indica* A. Juss), Schmutterer, H. and Ascher, K.R.S. and Rembold, H., Eds., GTZ press, Eschborn, West Germany,223.

Abid, M. and Maqbool, M.A. (1991), Effect of bare-root dip treatment in oil-cakes and neem leaf extract on the root-knot development and growth of tomato and eggplant, Pak. J. Nemat., 9, 13-16.

Adhikary, S., (1983) Results of field trials to control the diamondback moth, *Plutella xylostella* L., by application of crude methanolic extracts and aqueous suspensions of seed kernels and leaves of neem, *Azadirachta indica* A. Juss, in Togo, Z. Angew. Entomol., 100, 27.

Alam, M.M. (1991), Control of plant-parasitic nematodes with oilseed cakes on some vegetables in field, Pakistan J. Nemat., 9, 21-30.

Alder, V.E. and Uebel, E.C. (1984), Antifeedant bioassays of neem extract aganist the Carolina grasshopper, Walkingstick, and field cricket, J. Environ. Sci. Helth, 19, 393-403.

Aldhous, P. (1992) Neem chemical: The pieces fall into place , Science, 258,893.

Annadurai, R.S. and Rembold, H. (1993), Azadirachtin A modulates the tissue specific 2D polypeptides patterns of the locust, *Schistocerca gregatia,* Naturwissenschften, 80, 127-130.

Arpaia, A. and van Loon, J.J.A. (1993), Effects of azadirachtin after systematic uptake into *Brassica oleracea* L. on larvae of *Pieris brassicae* L., Entomologia exp. appl., 66, 39-45.

Arya, A. (1988), control of *Phomopsis* fruit rot by leaf extracts of certain medicinal plants, In Indigenous Medicinal Plants (Microbes and fungi), Eds. Kaushik, P., National Seminar, New Delhi, 41-46.

Attri, B.S. (1975), Utility of neem oil extractive as feeding deterrent to locusts, Indian J. Ent., 37(4), 414-419.

Ayyangar, G.S.G. and Rao, P.J. (1989), Azadirachtin effects on consumption and untilization of food and midgut enzymes of *spodoptera litura*, Indian J. Ent., 51, 373-376.

Azambuja, P., Garcia, E.S., Ratcliffe, N.A. and Warthen Jr, J.D. (1991), Immune-depression in *Rhodnius prolixus* induced by the gorwth inhibitor, azadirachtin, J. Insect Physiol., 37, 771-777.

Babu, T.H. and Beri, Y.P. (1969), Efficacy of neem (*Azadirachta indica* A. Juss) seed extracts in different solvents as deterrent to the larvae of *Euproctis lunata* Wlk., Andhra Agric. J., 16, 331-332.

Barker, G.C., Mercer, J.G., Svoboda, J.A., Thompson, M.J., Rees, H.H. and Howells, R.E. (1989), Effects of potential inhibitors on *Brugia pahangi in vitro* : macrofilaricidal action and inhibition of microfilarial production, Parasitology, 99, 409-416.

Barnby, M.A. and Klocke, J.A. (1987), Effects of azadirachtin on the nutrition and development of the tobacco budworm, *Heliothis virescens* (Fabr.), Noctuidae, J. Insect Physiol., 33, 69-75.

Barnby, M.A., Yamasaki, R.K., & Klocke, J.A., (1989a) Biological activity of azadirachtin, three derivatives, and their ultraviolet radiation degradation products aganist tobacco budworm (Lepidoptera: Noctuidae) larvae. J. Econ. Entomol. 82, 58-63.

Barnby, M.A.; Klocke, J.A.; Darlington, M.V. and Yamasaki, R.B. (1989b) Uptake metabolism and excretion of injected tritiated 22,23 dihydroazadirachtin in the last instar larvae of *Heliothis virscens,* Entoml. Exp. Appl., 52, 1-6.

Bassemir, U. (1977) Ultrasturcture differntiations in the developing follicle cortex of *Locusta migratoria* with special reference to vetellin membrane formation, Cell Tiss. Res., 185, 247-262.

Beckage, N.E., Metcalf, J.S., Nielson, B.D. and Nisbet, D.J. (1988), Distruptive effects of azadirachtin on development of *Cotesia congregata* in host tobacco hornworm larvae, Archs Insect Biochem. Physiol., 9, 47-65.

Benge, M.D. (1988) cultivation and propagation of the neem tree, In.M. Jacobson, (Ed), Focus on phytochemical Pesticides Vol.I, The neem Tree, CRC Press , Inc. Boca Raton, Florida.

Bernays,E.A. and Chapman, R.F. (1973) The regulation of feeding in *Locusta migretoria*: internal inhibitory mechanisms, Entomologia Experimentalis et Applicata, 16, 329-342.

Bidmon, H.-J., Kauser, G., Mobus, P. and Koolman, J. (1987), Effect of azadirachtin on blowfly larvae and pupae, In: Eds, Schmutterer, H., Ascher, K.R.S., Natural pesticides from the neem tree and other tropical plants; Proceedings of the Third International Neem Conference, GTZ, Eschborn, Germany, 232-272.

Bilton, J.N.; Broughton, H.B.; Jones, P.S.; Ley,S.V.; Lidert, Z.; Morgan,E.D.; Rzepa,P.N.; Scheppard, R.N.; Salvin,A.M.Z. and William, J.D., (1987), Structure reappraisal of limonoid insect antifeedant azadirachtin, Tetrahedron, 43, 2805,

Blaney, W.M. and Winstanley, C. (1980) Chemosensory mechanism of locusts in relation feeding: the role of some secondary plant compounds. Insect Neurobiology and Pesticide Action (Neurotox 1979), Society of chemical Industry, London, 383-389.

Borsdorf, R.; Foster, K.; Huyser, W.; Pedersen, J.; Pfost, H.; Stevens, H.; and Wright, V. (1983) Post-harvest management project design, Pakistan, Feed and Feed Grain Inst. Rep. No.91Manhattan, Kansas.

Bowers, W.S. (1976), Discovery of insect antiallatotropins, In " The Juvenile Hormones", Ed. L.I. Gilbert, 394-408, Plenum, New york.

Bowers, W.S. and Aldrich, J.R. (1980), *In vivo* inactivation of denervated corpora allata by precocene II in the bug *Oncopellus fasciatus*, Experientia, 36, 362-363.

Bowers, W.S. and Martinez-pardo, R, (1977) Antiallatotropins: Inhibition of corpus allatum development, Science, 197, 1369-1371.

Bownes, M. (1986) Three genes for three yolk proteins in *Drosophila melanogaster*, FEBS Lett., 200, 95-98.

Broughton, H.B., Ley, S.V., Slawin, A.M.Z., Williams, D.J. & Morgan, E.D. (1986) X-ray crystollographic structure determination of detigloyldihydro-azadirachtin and reassignment of structure of the limonoid insect antifeedant azadirachtin, J. Chem. Soc. Chem. Commun. 46-47.

Butterworth, J.H. and Morgan, E.D. (1968) Isolation of a substances that suppresses feeding in locust, J.Chem. Soc. Chem. Comm., 23-24.

Butterworth, J.H. and Morgan, E.D. (1971) Investigation of the locust feeding inhibition of the seeds of the neem tree,*Azadirachta indica*; J.Insect Physiology, 17, 969-977

Cardon, J.; Van Coillie, C.; Geysen, J. and De Loof, A. (1988) Yolk polypeptides synthesis in the fat body of *Sarcophaga bullata*; Localization,hormonal induction and cell-free translation, Insect Biochem., 18, 287-294.

Chari, M.S. and Murlidharan, C.M. (1985), Neem as feeding deterrent of castor semilooper, Achaea janata L. J. Entomol. Res., 9(2), 243-245.

Chavan S.R.(1984), Chemistry of alkane separated from leaves of *Azadirachta indica* and their larvicidal/ insecticidal activity against mosquitoes, In: Eds, Schmutterer, H., Ascher, K.R.S., Natural pesticides from the neem tree and other tropical plants; Proceedings of the Third International Neem Conference, GTZ, Eschborn, 59-66.

Chellayan, S. and Karnavar, G.K. (1990) Influence of neem kernel extract on morphogenesis and vitellogenic oocyte development in *Trogoderma granrium* everts, Proc. Indian Acad. Sci., 99, 113-118.

Chen, T.T.; Couble, P.; Abu-Hakima R. and Wyatt G.R. (1979) Juvenile hormone controlled vitellogenin synthesis in *Locusta migratoria* fat body, Devl. Biol. 69, 59-72.

Chen, T.T.; Couble, P.; de Lucca, F.L. and wyatt, G.R. (1976) Juvenile hormone control of vetellogenin synthesis in *Locusta migratoria* . In the Juvenile Hormones , Eds: Gilbert, L.I., 505-529, Plenum Press, New york.

Chen,A.C.; Kim, H.R.; Mayer, R.T. and Norman, J.O. (1987) Vitellogenin in the stablefly *Stomoxys calcirans*, Comp. Biochem. Physiol., 88B, 897-903.

Chinzei, Y.; White, B.N. and Wyatt, G.R. (1982) Vitellogenin mRNA in locust fat body; identification, isolation, and quantitative change induced by jevenile hormone, Can. J. Biochem, 60, 243-251.

Clemants, A.N. (1959) Studies on the metabolism of locust fat body, J. exp. Biol., 36, 665-675.

Coles, G.C. (1964) Some effect of decapitation on metabolism in *Rhodnius prolixux* , Nature, Lond, 203, 323.

Cottee, P.K. (1984), A physiological investigation into the role of secondary plant compounds as feeding deterrents, Ph.D. thesis, University of Aberdeen U.K.

Cotty, V.F.; Henry, M and Hilchey, J.D.; (1958) , Boyce Thom. Ins. Contr., 19, 379-392.

Devakumar, c. and Goswami, B.K. (1992) Nematicidal Principals from neem III , Isolation and Screeing of neem meliacins, Pescide Res. J. 4: 79-86.

Devi, D.A. and Mohandas, N. (1982), Relative efficacy of some antifeedants and deterrents against insect pests of stored paddy, Entomon 7, 261.

Dimetry, N.Z. and Schmidt, G.H. (1992), Efficacy of Neem-azal S and "Margosan-O" against the bean aphid, *Aphis fabae* Scop., Anz. Schadlingskde, Pflanzenschutz, Umweltschutz, 65, 75-79.

Doharey, K.L. and Singh, R.P. (1989), Evaluation of neem (*Azadirachta indica* A. Juss) seed kernel extract aganist chafer beetles, Indian J. Ent., 51(2), 217-220.

Dorn, A., Rademacher, J.M. and Sehn, E. (1986), Effects of azadirachtin on the moulting cycle, endocrine system and ovaries in last-instar larvae of the milkweed bug, *Oncopellus fasciatus*, J. insect physiol., 32, 321-328.

Dorn, A.; Ramecher, J.M. and Sehn, E. (1987) Effects of azadirachtin on reproductive organs and fertility in the large Milkweed bug *Oncopeltus fasciatus*, Proc. Third Int. Neem Conf. H. Schmutterer and K.R.S. Ascher (Eds.) GTZ, Eschborn, Germany.

Dow, J.A.T (1981) Countercurrent flows, water movements and nutrient absorbtion in the locust midgut, J. Insect Physiology, 27, 579-585.

Dreyer, M. (1987) Field and laboratory trials with simple neem products as protectants aganist pests of vegetable and field crops in Togo, in Proc. 3rd Int. Neem Conf., Nairobi, Kenya, Schumutterer, H. and Ascher, K. R. S., Eds., GTZ press, Eschborn, West Germany, 431.

Eder, U. and von Keyserlingk, H.C. (1985), The challenge of Finding New Insecticides for a mature Market, In: Eds., von Keyserlingk, H.C. and von Szczepanki, Ch., Approaches to New leads for Insecticides, Schering AG, Agricultural Reserch, Berlin, FRG, 1-8.

Ermel, K.; Pahlich, E. and Schimutterer, H. (1984) Comparision of azadirachtin content of neem seed from ecotypes of Asian and African origin. In H. Schmutterer and K.R.S. Aschen (eds), Natural Pesticides from the neem tree and other plants, Proceedings of the second International Neem Conference, GTZ, Eschborn, 587.

Ermel, K.; Pahlich, E. and Schimutterer, H. (1987) Azadirachtin content of neem kernels from different geogaphical locations and its dependence on temperature, relative humidity and light . In H. Schmutterer and K.R.S. Aschen (eds), Natural Pesticides from the neem tree and other plants, Proceedings of the second International Neem Conference, GTZ, Eschborn, 703.

Fagoonee, I. (1979) The potential of natural product in crop protection in Mauitius, In Proc. Neth. Agric. Prod. Conf., 1979, Reduit, Mauritius, 201.

Faulkner, P. and Bheemeswar, B. (1960) Studies on the biosynthesis of proteins in the silkworm *Bombyx mori* L., Biochem. J., 76, 71-78.

Ferenz, H.J.; Lubzenes, E. and Glass, H. (1981) Vitellin and vitellogenin incorporation by isolated oocytes of *Locusta migratoria* , J. Insect Physiol., 12, 869-875.

Fernando, S. (1982), Herbal Food and Medicine in Sri Lanka, The National NGO Council of Sri Lanka with support of FAO, Colombo, Sri Lanka, 114.

Feuerhake, K. and Schmutterer, H. (1985) Development of a crude standardized and fomulated insecticide from crude neem kernel extract, Z. Pflanzenkr. Pflanzenshutz, 92, 643.

Feurhake, K.J. (1984), Effectiveness and selectivity of technical solvents from the extraction of neem seed components with insectisidal activity, In: Natural pesticides from the neem tree and other topical plants, H. Schmutterer and K.R.S. Ascher (eds) Proc. 2nd Int. Neem Conf. Ranschholzha Usen, GTZ, Eschborn,FRG, 103-114.

Forgash, A.J. (1984), History, evalution and consequences of insecticides resistance, pestic. Biochem. Physiol., 22, 178-186.

Forster, H., (1983) Isolation of azadirachtin from Neem (*Azadirachta indica*) and radioactive labelling of azadirachtin, M.S. thesis, University of Munch, West Germany, 1983.

Fritzsche, U. and Cleffmann, G., (1987), The imsectidide azadirachtin reduces predominantly cellular RNA in *Tetrahymena*, Naturwissenschaften, 74, 191.

Garcia, E.S., Azambuja, P., Forster, H., and Rembold, H. (1984), Feeding and moult inhibition by azadirachtin A,B, and 7-acetylazadirachtin A in *Rhodnius prolixus* nymphs, Z. Naturforsch., 39c, 1155.

Garcia, E.S., Subrahmanyam, B., Muller, T. and Rembold, H. (1989), Absorption, storage, organ distribution and excretion of dietary ^{3}H-dihydroazadirachtin in *Rhodnius prolixus*, J. Insect Physiol., 35, 743-749.

Gellissen, G., and Waytt, G.R. (1981) Production of lipophorin in the fat body of adult *Locusta migratoria* : Comparision with vitelogenin, Can. J. Biochem., 59, 648-654.

Gellissen, G.; Waje, E.; Cohen, E.; Emmerich, H.; Applebaum, S. and Flassdorf, J. (1976) Purification and properties of oocyte vetellin from the migratory locust, J. Comp. Physiol., 108, 287-330.

Giles, P. H. (1964) The storage of cerels by farmers in Northern Nigeria, Trop. Agric., 41, 197.

Gill, J.S. and Lewis, C.T. (1971), Systemic action of an insect feeding deterrent, Nature, 232, 402-403.

Girardie, J. ; Faddoul, A. and Girardie, A. (1985) Characterization of three neurosecretory proteins from the A median neurosecretory cells of *Lcusta migratoria* by coupled chromatographic, electrophoretic and isoelectrofocusing methods, Insect Biochem., 15, 85-92.

Girardie, J.; Boureme, D. and Girardie, A. (1987a) Production sites of the three neurosecretory proteins characterized in the corpora cardiaca of the migratory locust, Insect Biochem., 17, 29-36.

Girardie, J.; Boureme, D.; Couillaud, F.; Tamarelle, M. and Girardie, A. (1987b) Anti juvenile effect of neuroparsin A a neuroprotein isolated from locust copus cardiaca, Insect Biochem., 17, 977-983.

Girardie, J.; Richard, O. and Grardie, A. (1992) Time-dependent variations in the activity of a novel ovary maturing neurohormone from the nervous corpora cardiaca during oogenesis in the locust *Locast migratoria* , J. Insect Physiol., 38, 215-221.

Gokte, N. and Swarup, G. (1988), Effect of neem (*Azadirachta indica*) seed kernel powder treatment on penetration and development of *Anguina tritici* on wheat, Indian J. Nematol., 18, 149.

Goldworthy, G.J.,(1969) Hypercaemic factors from the corpus cardiacum of *locusta migratoria*, J. Insect Physiology, 15,2131.

Goltzene, F. and Porte, A. (1978) endocrine control by neurosecretory cells of the pars intercebralis and the corpora allata during the earlier phases of vitellogenesis in *Locusta migratoria* , Gen. Comp. Endocr., 35, 35-45.

Govindachari, T.R., Sandhaya, G. and Ganeshraj, S.P. (1992) Journel of Natural Products, 55, 5, 596-601.

Govindachari, T.R., Sandhya, G. and Ganeshraj, S.P. (1990), Simple method for the isolation of azadirachtin by preparative HPLC, J. Chrom. , 513, 389-391.

Gow, J.A.T. (1981) Countercurrent flows, water movements and nutrient absorbtion in the locust midgut, J. Insect Physiology, 27, 579-585.

Griffiths, D.C., Greenway, A.R. and Lloyd, S.L. (1978), The influence of repellent materials and aphid extracts on setting behaviour and larviposition of Myzus persicae (Sulzer) (Hemiptera: Apjididae). Bull. Ent. Res., 68, 613-619.

Gujar, G.T. and Mehrotra, K.N. (1984), Inhibition of growth and development of the tobacco caterpillar, *Spodoptera litura* Fab. due to azadirachtin and other neem products, Indian J. Ent., 45(4), 431-435.

Gupta, H.C., Verma, J.P., Bareth, S.S. and Mathur, B.N. (1989), Evaluation of some non-edible oils as grain protectant in wheat and their subsequent effect on germination, Indian J. Ent., 50, 147-150.

Hagedorn, H.H. (1985) The role of ecdysteroids in reproduction. In " Physiology, Biochemistry and Pharmacology", Eds: Kerkut, G.A. and Gilbert, L.I., 8, 205-261, Pergamon Press, Oxford.

Hagedorn, H.H. and Kunkel, J.G. (1979) Vitellogenin and vitellin in insects, Annu. Rev. Entomol., 24, 475-505.

Harnish, G.D. and White, B.N. (1982) Insec vitellins: Identification, Purification and characterization from eight orders, J. Exp. Zool., 220, 1-10.

Harwood,L.M.(1985),"Dry-column" flash chromatography,Aldrichchemica Acta, 18,25.

Helliwell, R.M. and Mordue, A.J. (1991), Does azadirachtin affect insect visceral muscle?, Neurotox '91 - An International Symposium, Pest. Sci., 33, 115-116.

Henrick, C.A.; StallG.B. and Siddall, J.B. (1973) Alkyl 3,7,11- trimethyl-2,4-dodecadienoates, a new class of protein insect growth regulators with juvenile hormone activity, J. Agr. Food Chem., 21, 354-359.

Highnam K.C. (1963) Neurosecretory control of ovarian development in *schistocerca gregaria*, Quart. J. micr. Sci., 103, 57-72.

Highnam, K.C. (1962b) Neursecretory control of ovarian development in the desert locust, Mem. Soc. Endocrin., 12, 379-390.

Highnam, K.C. (1962 a) Neurosecretory control of ovarian development in *Schistocerca gregaria*, Quart. J. micr. Sci., 103, 57-72.

Highnam, K.C. and Lusis O. (1962) The influence of mature male on the neurosecretory control of ovarian development in the desert locust, Quart. J. micr. Sci., 103, 73-83.

Highnam, K.C. and Mordue (Luntz) A.J. (1974) Induced changes in neurosecretory activity of adult female *Schistocerca gregaria* in relation to feeding, Gen. Comp. Ecdor., 22, 519-525.

Highnam, K.C. and Mordue, W. (1966) The endocrine system and oocyte growth in *Schistecerca* in relation to starvation and frontal ganglianectomy, J. Insect Physiol., 12, 977-994.

Highnam, K.C., Lusis, O. and Hill, L. (1963) The role of the corpora allata during oocyte development in *Schstocerca gregaria* , J. Insect Physiol., 9, 587-596.

Hill, L. (1962) Neurosecretory control of haemolymph protein concentration during ovarian development in the desert locust, J. Insect Physiology, 8, 609-619.

Hill, L. (1965), The incorporation of C^{14}-glycine into the proteins of the fat body of the desert locust during ovarian development, J. Insect Physiol., 11, 1605-1615.

Hongo, H. and Karel, A.K. (1986) Effect of plant extract on insect pests of common beans, J. Appl. Entomol., 102, 164.

Imboden, H. and Low, J.H. (1983) Heterogenity of vitellins and vitellogenins of the tobacco hornworm, *Manduca sexta* L., Time course of vitellogenins appearnce in the haemolymph of the adult female, Insect Biochem., 13, 151-162.

Isman, M.B., Koul, O., Luczynski, A. & Kaminski, J., (1990) Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content, J. Agric. Food Chem., 38, 1406-1411.

Ivbijaro, M.F. (1990), The efficacy of seed oil of *Azadirachta indica* A. Juss and *Piper guineense* a neem-based insecticide for Canada, Mem. ent. Soc. Can., 159, 39-47.

Jacobson, M. (1986), The neem tree: Natural par excellence, In: Eds, Green, M.B. and Hedin, P.A., Natural resistance of plants to pests; roles of allelochemicals, ACS symposium seris 296, American Chemical Society, Washington DC.

Jacobson, M. (1988) Focus on phytochemical pesticides , 1,CRC Press Inc., Boca Raton, Florida.

Jagannathan, R. and Narasimhan, V. (1988), Effect of plant extracts/ products on two fungal pathogens of finger miller, Indian J. Mycol. Plant Path., 18, 250-254.

Jain, H.K.; Srivastava, K.P.; Agnihotri, N.P. and Cajbhiye, V.T. (1986) Evaluation of bioeffecacy of some insecticides aganist *Heliothis armigera* and *Melanagromyza obtusa* their residues on pigeon pea, Int. Pigeonpea Newslett.

Johnson, R.A. and Hill, L. (1975) Activity of the corpora allata in the adult female migratory locust, J. Insect Physiol., 21, 1517-1519.

Jone, P.S.; Ley, S.V. ; Morgan,E.D. and Santafianos, D., (1988) The Chemistry of the neem tree, In. M. Jacobson (ed), Focus on Phytochemical pesticides Vol.1 The neem tree, CRC Press Inc., Boca Raton, Florida.

Jotwani, M.G. and Sircar, P. (1965), Neem as protectant against stored grain pests infesting wheat seed, India J. Ent., 27(2), 160-164.

Jotwani, M.G. and Sircar, P. (1965), Neem as protectant against stored grain pests infesting wheat seed, Indian J. Emt., 27(2), 160-164.

Jotwani, M.G. and Sircar, P. (1967), Neem as protectant against bruchid, *Callosobruchus maculatus* (Fabricius) infesting some legumes, Indian J. Ent., 29(1), 21-24.

Kanost, M.R., Kawooya, J.K., Law, J.H., Ryan, R.O., Van Heusden, M.C. and Ziegler, R. (1990) Insect Haemolymph Proteins, In: Advances in Insect Physiology, Eds., Evans,P.D. and Wigglesworth, V.B., Academic Press, New York, 22, 323.

Kareem, A, Saxena, R.C. and Boncodin, M.E.M. (1988), Neem seed kernel extract and neem bitters affect oviposition and hatchability of eggs *Niphotettix virescens*, Neem Newsletter, 5(1), 9-10.

Karel, A.K. (1987) Response of *Ootheca bennigseni* (Coleoptera: Chrysomelidae) to neem extracts, in Proc. 3rd Int. Neem Conf., Nairobi, Kenya, Schumutterer, H. and Ascher, K. R. S., Eds., GTZ press, Eschborn, West Germany, 393.

Karnavar, G.K. (1987), Influence of azadirachtin on insect nutrition and reproduction. Proc. Indian Acad. Sci. (Anim. Sci.), 86, 431-347.

Karus, W., Baumann, S., Bokel, M., Keller, U., Klenk, A., Klingele, M., Pohnl, H. and Schwinger, M. (1987), Control of insect feeding and development by constituents of *Melia azaderach* and *Azadirachta indica*, In. Eds, Schmutterer, H., Ascher, K.R.S., Natural pesticides from the neem tree and other tropical plants; Proceedings of the Third International Neem Conference, GTZ, Eschborn, Germany, 703.

Kataoka, H.; Troestschler, ar.g.a; Li, J.P.; Kramer, S.J.; Carney, R.L. and Schooley, D.A. (1989) Isolation and identification of diuretic hormones from the tobacco hornworm, *Manduca sexta*, Proc. nat. Acad. Sci. U.S.A., 86, 2976-2980.

Kauser, G. and Koolman, J. (1984) Ecdysteroid receptor in tissues of the blowfly, *Callliphora vicina,* in Advances in Invertibrate Reproduction, vol.3, Engels, W.(ed) Elsevier, Amsterdam, 602.

Keeley, L.L. and McKercher, S.C. (1985) Endocrine regulations of ovarian maturation in the cockroach *Blaberus discoidalis*, Comp. Biochem. Physiol., 80A, 115-121.

Khan, M., Schneider, B., Wassilew, S.W. and Splnemann, V. (1988), The effect of raw materials of the neem tree, neem oils and neem extracts of dermatophytes, yeasts and molds, Z. Hautke, 63, 499-502.

Kilby, B.A. and Neville, E. (1957) Amino acids metabolism in locust tissues., J. Exp. Biol., 34, 276-289.

Kirsch, K., (1987) Studies on the effecacy of neem extracts in controlling major pests in tobacco and cabbage, Proc. 3rd Int. neem Conf. Nairobi, Kenya, Eds. Schumutterer, H. and Ascher, A.R.S., GTZ Press Eschborn, West Germany, 495.

Klocke, H.A. and Kubo, I. (1991), Defense of plants through regulation of insect feeding behaviour, Fla Ent., 74, 18-23.

Koul, O. (1984), Azadirachtin II. Interaction with the reproductive behaviour of red bug, Z. angew. Ent., 98, 221-223.

Koul, O., Amanai, K. and ohtaki, T. (1987) Effect of azadirachtin on the endocrine events of *Bombyx mori*, J. Insect Physiol., 33, 103-108.

Koul, O., and Isman, M.B. (1991), Effects of azadirachtin on the dietary utilization and development of the variegated cutworm *Peridroma saucia*, J. insect physiol., 37, 591-598.

Kraus, W., Bokel, M., Bruhn, A., Cramer, R.; Klaiber, I.; Klenk, A., Nagl, G., Pohnl, H., and Vogler, B. (1987) Structure determination by NMR of azadirachtin and related compounds from *Azadirachta indica* A Juss (Meliaceae), Tetrahedron, 43, 2789.

Kraus, W., Bokel, M., Klenk, A., and Pohnl, H. (1985), The structure of azadirachtin and 22,23 dihydro 23ß, methoxyazadirachtin, Tetrahedron Lett., 26, 6433-6438.

Kubo.I., Matsumoto,A., and Matsumoto, J. (1986) New insect ecdysis inhibitoy limonoid deacetylazadirachtinol isolated from *A. indica* oil, Tetrahedron,42,2,489-496.

Kunkel, J.G. and Nordin, J.H. (1985) Yolk proteins, In " Comprehensive Insect Physiology, Biochemistry and Pharmacology", Eds: Kerkut, G.A. and Gilbert, L.I., 1, 83-111, Pergamon Press, Oxford.

Lamy, M. (1984) Vitellogenesis, vitellogenin and vitellin in the males of insects: A reiview, Int. J. Invert. Reprod. and Develop., 7, 311-321.

Larson, R. O. (1987) Development of Margasan-O, a pesticide from neem seed, in Proc. 3rd Int. Neem Conf., Nairobi, Kenya, Schumutterer, H. and Ascher, K. R. S., Eds., GTZ press, Eschborn, West Germany, 243.

Ley, S.V., Denholm, A.A. and Wood, A. (1993), The chemistry of azadirachtin, Nat. Products Reoprts., 109-157.

Loughton, B.G. and Tobe, S.S. (1969) Blood volume in the African migratory locust; Canadian Journal of Zoology, 47, 1333-1336.

Lowry, O.H.; Rosebrough, N.J.;Farr,A.L. and Randall, R.J.(1951) Protein measurement with the folin phenol reagent, J. Bio. Chem. 193, 265-275.

Macro, M-P; Pascual, N.; Belles, X; Camps, F. and Messeguer, A. (1990) Ecdysteriod depletion by azadirachtin in *Tenebrio molitor* pupae, Pesticide Biochem. and Physiol., 38, 60-65.

Maddrell, S.H.P. The function of insect Malpighian tubules (eds. Bolis,L.; Keynes, R.D. and Wilbrandt, W.), Amsterdam, North Holland.

Maddrell, S.H.P.; Gardiner, B.O.C.; Pilcher, D.E.M. and Reynolds, S.E.(1974) Active transport by insect Malpighian tubules of acidic dyes and of acylamides, J. exp. Biol., 61, 357-377.

Maddrell, S.H.P.; Klunsuwan, S. (1973) Fluid secretion by *in vitro* preparation of the Malpighian tubules of the desert locust *S. gregaria.*,J, Insect Physiology, 19, 1369-1376.

Maddrell, S.H.P.; Pilcher, D.E.M. and Gardiner, B.O.C. (1971) Pharmocology of the Malpighian tubules of *Rhonius* and *Carausius*: the structure activity relation ship of tryptamine analogues and the role of cyclic AMP. J. Exp. Biol., 54, 779-804.

Makanjuola, W.A. (1989), Evaluation of extracts of neem (*Azadirachta indica* A. Juss) for the control of some stored product pests, J. Stored Prod. Res., 25, 231-238.

Malczewsla, M.; Gelman, D.B. and Cymborowski, B. (1988) Effects of azadirachtin on development, juvenile hormone and ecdysteroid titres in chilled *Galleria mellonella* larvae, J. Insect physiol., 34, 725-732.

Mane, S.D. (1968), Neem seed spray as repellent against some of the foliage feeding insects, M.Sc. Thesism Post graguate school, Indian Agriculture Research Institute, New Delhi.

Mansour, S. A. and Ascher, K.R.S., Eds.(1984) Effects of neem (Azadirachta indica A Juss) seed kernel extracts from different solvents on the carmine spider mite. Tetranychus urticae, in Natural Pesticides from the Neem Tree and Other Tropical Plants, Schmutterer, H. and Ascher, K. R. S. and Rembold, H.,Eds., GTZ press, Eschborn, West Germany,461.

McGregor, D.A. and Laughton,B.G. (1977) Amino acid composition, degradation and utilization of locust vitellogenin during embryogenesis, wilhelm Roux Arch. Entw. Mech. Org., 181, 113-122.

McMillian, W.W., Bowman, M.C., Burton R.L., Stark, K.J. and Wiseman, B.R. (1969), Extracts of Chinaberry leaf as feeding deterrent and growth retardant for larvae of corn earworm and fall armyworm, J. Econ. Entomol., 62, 708-710.

Meisner, J., Klein, M. and Keren, S. (1990), Effect of "Margosan-O" on the development of *Earial insulana*, Phytoparasitica, 18, 287-297.

Meisner, J., Klein, M., and Ben-Moshe, E. (1992), Effect of "Margosan-O" on the development of leafhopper *Asymmertrasca decedens*, Phytoparacitica, 20, 15-23.

Morallo-Rejesus, B., Maini, H.A., Ohsawa, K., yamamoto, I. and Mitchel, R. (1990), Insecticidal actions of several plants to Callosobruchus chinensis L., In: Bruchids and Legumens, Economics, Ecology, and Evolution, Proc. 2nd Int. Symp. (ISBL-2), Okayama, Japan, Ser. Ent., 46, 91-100.

Mordue (Luntz), A.J.; cottee, P.K. and Evans K.A. (1985) Azadirachtin: its effect on gut mortility, growth and moulting in *Locusta,* Physiol. Entomo., 10, 431-437.

Mordue, A.J. and Blackwell, A. (1993), Azadirachtin update, J. Insect Physiol., 39, 903-924.

Mordue, A.J. and Highnum, K.C. (1973) Incorporation of cysteine into the cerebral neurosecretory system of adult desert locusts, Gen. comp. Endor., 20, 351-357.

Mordue, A.J.; Evans, K.A. and Charlet, M. (1986) Azadirachtin, ecdysteroids and edcysis in *Locusta migretoria* , Comp. biochem. physiol., 85C, 297-301.

Mordue, W. (1966) Hormonal control of Malphigian tube rectal function in the desert locust, *Schistocerca gregaria*, J. Insect Physiol., 15, 273-285.

Mordue, W. (1968) Hormones control of Malpighian tubules and rectal function in the desert locust *S. gregaria*, J. Insect Physiol., 15, 273-285.

Mordue, W. (1980) Terrestial insects-hormones and water balance, J. Insect Physiology, 26,357-377.

Mordue. W. (1969) Possible Mode of action of the diuretic water balance, J. Insect Physiology, 15,24.

Morgan, P.J.and Mordue, W. (1985) Cyclic AMP and locust diuretic hormones action, Insect Biochemistry, 15, 247-257.

Mundall, E.C. and Law, J.H. (1979) Physical and chemical characterization of vitellogenin from the haemolymph and eggs of the tobacco hornworm, *Manduca sexta,* Comp. Biochem. Physiol., 63B, 459-468.

Muthusamy, M., Eswaramurthy, S., Muthusamy, S. and Mrappan, V. (1988), Evaluation of neem products aganist rust disease of groundnut, Neem Newsl., 5, 48.

Nakanishi, K.; (1975); Advances in Phytochemistry Ed. by V.C. Runeckles, Plenum press, New york, 9, Chapter 11.

Naqvi, S.N.H., Tabassum, R., Zia, N. and Nurulain, S.M. (1990), Toxicity and residual effect of neem extract (factor C) against stored grain pest *Callosobruchus analis*, Pakistan J. Zool., 22, 271-277.

Nasiruddin, M. and Mordue (Luntz), A.J.(1993) The protection of barley seedling from attack by *Schistocerca gregaria* using azadirachtin and related analogues, Entomol. Exp. appl.

Naumann, K. (1994) Recent Botanical Insecticides Research and Practicle Application, New Strategies for Locust Control, In Natural Product and Receptor Reserch, Eds. Rembold, H., Benson, J.A., Franzen, H., Weickel, B. and Schulz, F.A., Pro. of the CEC work shop, Humburg, Germany, 14.

Newton, G.L., Aguilera, J.A., Fahey, R.C., Ward, J.F., Radkowasky, A.E. and Kosower, E.M. (1992) para-Sulfobenzoyloxybromobimane: A new membrane Impermeable reagent useful for the analysis of thiols and their export from cells, Analytical Biochemistry, 201, 30-42.

Nisbet, A.J. (1992), The effects of azadirachtin on the feeding behaviour and virus transmission of the Green peach aphid *Myzus persicae* (Sulzer), Ph.D. thesis, University of Glasgow, U.k.

Nisbet, A.J., Woodford, J.A.T., Strang, R.H.C., and Connolly, J.D. (1993), Systemic antifeedant effects of azadirachtin on the peach-potato aphid *Myzus persicae*, Entomologia exp. appl., 68, 87-98.

Norris, D.M. (1986) Antifeeding compounds, In: Chemistry of Plant Protection, Eds. Haug, G. and Hoffman, H., Springler-Verlag, Berlin, Vol. 1, 97-146.

Parmer, B.S. and Ketkar, C.M. (1993) Commercialization, Neem Research and Development, Eds. Randhawa, N.S. & Parmar, B.S., No.3, Society of Pesticide Science, India, 270-283.

Parmer, B.S.(1987) An overview of neem research and use in India during the years 1983-1986, in Proc. 3rd Int. Neem Conf. Nairobi, Kenya, Schmutterer, H. and Ascher, K. R. S.,Eds., GTZ press, Eschborn, West Germany, 55.

Pathak, P.H. and Krishna, S.S. (1985), Neem seed oil, a capable ingredient to check rice moth reproduction (Lepid. Galleriidae), Z. angew. Ent., 100, 33-35.

Pener, M.P. and Shalom, V. (1987), Endocrine manipulations, juvenile hormone and ontogenesis of male sexual behaviour in locusts, Insect. Biochem., 17, 1109-1113.

Pener, M.P., Van den Broek, A.T.M., Van Marrewijik, W.J.A., Van Koorn, J.M., Vander Horst, D.J. and Beenakkers, A.M.T. (1989), Development of imaginal competence to adipokinetic hormone in *locesta*: lipid and carbohydrate and glycogen phosphorylase activity in azadirachtin-induced over-aged nymphs, Comp. Biochem. Physiol., 94B, 293-298.

Pener, M.P.; Orshan, L. and de Wilde, J. (1978) Precocene II causes atrophy of corpora allata in *Locusta migratoria*, Nature, London , 72, 350-353.

Phillips, J.E. (1980), Epithelial transport and control in recta of terrestral insects, In: Insect Biology in the future (Ed. Locke, M.L. and Smith, D.S.), Academic Press, New York, 145-177.

Phillips, J.E. (1981), Comparative physiology of insect renal function, Am. J. Physiol., 241, R241-R257.

Price, J.F., Schuster, D. J. and McClain, P.M. (1990) Azadirachtin from neem tree (*Azadirachta indica* A. Juss) seed for management of sweetpotato whitefly [*Bermisia tabaci* (Gennadius)] on ornametals, Proc. Fla State Hort. Soc. 103, 186-188.

Rafaeli, A.; Pines, M.; Stren, P.S. and Applebaum, S.W. (1984) Locust diuretic hormone-stimulated synthesis and excretion of cAMP; a novel Malpighian tubules bioassay, Gen. Comp. Endor., 54, 35-42.

Ranaginghe, M.A.S.K. (1984) Neem and other promising botanical pest control materials from Sri Lanka, in Proc. Int. Workshop on the Use of Botanical Pesticides, International Rice Research Institute, Manila.

Randhawa, N.S. & Parmar, B.S. (1993), Introductory, Neem Research and Development, Eds. Randhawa, N.S. & Parmar, B.S., No.3, Society of Pesticide Science, India, 1-5.

Rednap, R. S. (1981) The use of crushed neem berries in the control of some insect pests in Gambia, in Natural Pesticides from the Neem Tree *(Azadirachta indica* A Juss), Schmutterer, H. and Ascher, K. R. S. and Rembold, H.,Eds., GTZ press, Eschborn, West Germany, 205.

Reed, D.K.; Warthen, J.D.; Jr. Ubel, E.C. and Reed, G.L. (1982) Effects of two triterpenoids from on feeding by cucumber beetles (Coleoptera: Chrysomelidae), J. Econ. Entoml., 75, 1109.

Rembold, H. (1981) Modulation of JH-III titre during the gonotrophic cycle of *Locusta migratoria*, measured by gas chromatography selected ion monitoring mass spectrometry, In "Juvenile Hormone Biochemistry", Eds. G.E. Pratt and G.T. Brooks, 11-20, Elsevier/ North-Holland, Amsterdam.

Rembold, H. (1988), Isomeric azadirachtin and their mode of action, In: Ed. Jacobson, M., Focus on phytochemical pesticides, Vol. 1 The Neem Tree, CRC Press, Inc., Boca Raton, Florida.

Rembold, H. and Sieber, K.-P. (1981) Inhibition of oogenesis and ovarian ecdysteriod synthesis by azadirachtin in *Locusta migratoria migretorioides* (R&F), Z. Naturforsch., 36c, 466-469.

Rembold, H.; and Subramanyam, B. and Muller, T. (1989) Copus cardiacum - a target for azadirachtin. Experientia, 45, 361- 363.

Rembold, H.; forster, H.; Czoppelt, C.; Rao P.J. and Sieber, K.-P. (1983) The azadirachtins, a group of insect growth regulators from the neem tree, *Azadirachta indica*, In Natural Pesticides from the Neem Tree and other Tropical Plants, Eds: Schmutterer, H. and Ascher, K.R.S., GTZ, Eschborn, 153-161.

Rembold, H.; Forster,H.; Czoppelt, ch.; Rao, P.J.; and Sieber, K.P.; (1984) the azadirachtins, a group of insect growth regulators from the neem tree, in Natural Pesticides from the Neem Tree and other tropical plants, Ed. Schmutter, H. and Ascher, K.R.S., GTZ press, Eschborn, West Germany, 153.

Rembold, H.; Muller, Th. and Subramanyam, B. (1988) Tissue- specific incorporation of azadirachtin in the malpighian tubules of *Locusta migratoria*, Z. Naturforsch,43c,903-907.

Rembold, H.; Uhl, M. and Muller, T. (1987) Effect of azadirachtin A on hormone titers during the gonadotrophic cycle of *Locusta migratoria* , In Natural lPesticides from the Neem Tree and Other Tropical Plants, Eds: Schmutterer, H. and Ascher, K.R.S., GTZ, Eschborn, 289-298.

Reynolds, S.E. and Wing, K.D. (1986) Interactions between azadirachtin and ecdusteroid-dependant systems in the tobacco hornworm, *Manduca sexta*, Abstracts Vth Int. Congr. Pest. Chem., IUPAC, Ottowa, Canada, P.2D/E-08.

Riddford, L.M. (1981), Hormonal control of epidermal cell development, Am. Zool., 21, 751-762.

Rossner, J. and Zebitz, C.P.W. (1987), Effect of neem products on nematodes and growth of tomato plants, In: Natural pesticides from the neem tree (*Azadirachta indica* A. Juss.) and other tropical plants, Eds. Shumutterer, Hl and Ascher, K.R.S., GTZ Press Eschborn, West Germany, 611-621.

Ruscoe, C. N. E. (1972) Growth disruption effects of an insect antifeedant, Nature New Biol., 236, 159-160.

Ruskin, F.R. (Ed), (1992),"Neem" A tree for solving global problems, National academy press, Washington, D.C., 1-14.

Sandhu, G.S. and Singh, D. (1975), Studies on antifeedant and insecticidal properties of neem, *Azadirachta indica* A. Juss and dharek *Melia azedarach* L. Kernel / fruit powder to *Pieris brassicae*, Indian J. Plant Prot., 3(2), 177-180.

Saxena, R.C. and Khan, Z.R. (1984), Servival of brown plant-hopper and its ability to trnsmit grassy stunt and ragged stunt diseases on neem oil-treated rice seedlings, Neem Newsl., 1, 25-27.

Saxena, R.C. Liquido, N.J. and Justo, Jr.H.D. (1981) Neem seed oil, a potential antifeedant for the control of rice brown planthopper, *Nilaparvata lugens,* In: Natural pesticides from neem tree, *Azadirachta indica* A. Juss., Eds. Schmutterer, H., Ascher, K.R.S. & Rembold, H., Proc. 1st Intl. Neem conf., Rottach Egern, GTZ Press, Eschborn,West Germany, 171-188.

Schluter, U. (1987), Effects of azadirachtin on developing tissues of various insect larvae, In: Eds, Schmutterer, H., Ascher, K.R.S., Natural pesticides from the neem tree and other tropical plants; Proceedings of the Third International Neem Conference, GTZ, Eschborn, Germany, 331-348.

Schluter, U.; Bidmon, H.J. and Grewe, S. (1985) Azadirachtin effects growth and endocrine events in larvae of the tobacco hornworm *Manduca sexta,* J. Insect Physiol., 31, 773-777.

Schmutterer, H. (1985), Which insect pests can be controlled by application of neem seed kernel extracts under field conditions?, Z. angew. Ent., 100, 468-475.

Schmutterer, H. (1988) Potential of azadirachtin-containing pesticides for integrated pest control in developing and industrialised countries, J. Insect Physio., 34, 713-719.

Schmutterer, H. (1990), Properties and potential of natural pesticides from the neem tree, *Azadirachta indica,* A. Rev. Ent., 35, 271-297.

Schmutterer, H. (1992), Einfluß von azadirachtin, einer azadiracnbtin-freien fraktion eines alkoholischen niemsamenextraktes und von formulierten extrakten auf verpuppung, schlupf und imagines der kohlweißlingsbrackwespe *Apanteles glomeratus* (L) (Hym., Braconidae), J. app. Ent., 113, 79-87.

Schmutterer, H. and Zebitz, C.P.W. (1984), effect of methanolic extracts from seeds of single neem trees of African and Asian origin, on Epilichna varivestis and aedes aegypti. In: H. Schmutterer and K.R.S. Ascher (eds), Natural pesticides from the neem and other tropical plants; Proceedings of the Second International Neem Conference, GTZ, Eschborn, 587.

Schmutterer, H., and Ascher, K.R.S. (Eds) (1987), Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants, Proc. 3rd Int. Neem Conf., GTZ, Eschborn, Germany.

Schoonhoven, L.M. (1982), Biological aspects of antifeedants, Entomol. exp. appl., 31, 57-69.

Schroeder, D.R. and Nakanishi, K. (1987) A simplified isolation procedure for azadirachtin, J. Nat. Prod., 50, 2, 241-244.

Shultz, E.B., Bhatangar, D., Jacobson, M., Saxena, R.L. & Unander, D., (1992) Neem , A Tree for Solving Global Problems, Eds. Ruskin, F.R.Report of an Ad Hoc Panel of the Board on Science and Technology for International Development National Reserch Council, National Acadamy Press, Washington.

Siddiqui, M.A. and Alam, M.M. (1990), Control of root-knot, reniform and stunt nematodes with nimbin seed dressing, Nematol. medit., 18, 19-22.

Sieber, K.-P. and Rembold, H. (1987) The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria* , J. Insect Physiol., 29,523-527.

Sieber, K.P. and Rembold, H. (1983), The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria*, J. Insect Physiol., 29, 523-527.

Simmonds, M.S.J. and Blaney, W.M. (1984), Some neurophysiological effects of azadirachtin on lepidopterous larvae and their feeding reponse, In: Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants, (Eds) Schmutterer, H., and Ascher, K.R.S., Proc. 2nd Int. Neem Conf., GTZ, Eschborn, Germany, 587.

Simpson, S.J. (1983) The role of volumenric feed-back from the hindgut in the regulation of meal size in fifth-instar *Locusta migratoria* nymphs, Physiol. Entomo., 8, 451-467.

Singh, B. and Singh, A.P. (1987), Neem seed kernel suspension as an oviposition deterrent to the desert locust, *Schistocerca gregaria* F., Plant Prot. Bull., 39,9-16.

Singh, K. and Sharma, U.L. (1986), Studies on antifeedant and repellent qualities of neem (*Azadirachta indica*) aganist aphid (*Brevicorne brassicae*) on cauliflower and cabbage, Res. Dev. Reptr., 3, 33-35.

Singh, R.P. (1984), Effect of water extract of deoiled neem kernel on second instar loarvae of *Culex fatigans* Wiedemann. Neem Newsletter, 1(2), 16-17.

Singh, R.P. (1987), Comparision of antifeedant efficacy and extract yields from different parts and ecotypes of neem (*Azadirachta indica* A.Juss), In: Eds, Schmutterer, H., Ascher, K.R.S., Natural pesticides from the neem tree and other tropical plants; Proceedings of the Third International Neem Conference, GTZ, Eschborn,185-194.

Singh, R.P. (1993), Bioactivity aganist insect pests, Neem Reasch and Development, Eds. Randhawa, N.S. and Parmar, B.S., Society of pesticides science, India, 3, 109-122.

Singh, R.P. and Kataria, P.K. (1986), Deoiled neem kernel powder as protectant of wheat seed against, *Trogoderma granarium*, Indian J. Ent., 48(1), 119-120.

Singh, R.P., and Srivastava, B.G. (1983), Alcohol extract of neem (*Azadirachta indica* A. Juss) seed oil as oviposition deterrent for *Dacus cucurbitae* Coq., Indian J. Ent., 45(4), 497-498.

Skinner, D.M. (1963) Incorporation of labeled valine into the proteins of the Cecropia silkworm, Biol. Bull., Woods Hole, 125, 165-176.

Sloper, J.C. (1957) Presence of a substance rich in protein bound cystine or cysteine in the neurosecretory system of and insect, Nature, 179, 148-149.

Smith, S.L. and Mitchell, M.J. (1988), Effects of azadirachtin on insect cytocrome P-450 depndant ecdysone 20-mono oxygenase activity, Biochem. biophys. Res. Commun. 154, 559-563.

Sridhar, S. and Chetty, J.S. (1989), Effects of *Azadirachta indica* and *Ponamia glabra* leaf extracts on food utilization and modulation of efficiency of digestive enzymes in *Euproctic fratera*, Proc Indian Acad. Sci., 98, 313-323.

Srivastava, K. P.; Agnihotri, N.P.; Gajbhiye, V.T. and Jain, H.K. (1984) Relative effecacy of fenvalerate, Quinalphos and neem kernel extracts for the control of pod fly, *Melanagromyza obtusa* (Malloch) and pod borer, *Heliothis armigera* (Hubner) infesting red gram, Cajanus cajan (L.) Millisp. together with their residues, J. Entomol. Res., 8,1.

Steets, R. (1976) The effects of crude of extracts from the meliaceous plants *Azadirachta indica* and *Melia azedarach* on some insect species, A. Angew. Entomol., 77, 306.

Still, W.C.; Kahn,M.; and Mitra, A. (1978), J. Organic Chemistry, 43, 2923.

Stokes, J.B. & Redfern, R.E. (1982) Effect of sunlight on azadirachtin: antifeedant potency, J. Environ. Sci. Hlth, 17, 57-65.

Subrahmanyam, B. and Rembold, H. (1989a) Effect of azadirachtin A on neuroendocrine activity in *Locusta migratoria*, Cell Tissue Res., 256, 513-517.

Subrahmanyam, S.; Muller, T. and Rembold, H. (1989b) Inhibition of turnover of neurosecretion by azadirachtin in *Locusta migretoria* , J. Insect Physiol., 35, 6, 493-500.

Tanzubil, A.W.; Strunz, G.M.; Chiasson, M. and Chan, T.H. (1990) Potential of Margosan O, an azadirachtin-containing formulation from neem seed extract, as a control agent for sprucre budworm, *Choristoneura fumiferana,* Ent. Exp. appl., 62, 37-46.

Taylor, D. A. H. (1987) Azadirachtin a study in the methology (sic) of structure determination, Tetrahedron, 43, 2779-2787.

Telfer, W.H. (1960) The selective accumulation of blood proteins by the oocytes of saturniid moths, Bilo. Bull. mar. biol. Lab. Woods Hole, 118, 338-351.

Telfer, W.H. (1965) the mechanism and control of yolk formation, A Rev. Ent., 10, 161-184.

Telfer, W.H. and Melius, M.E. (1963) The mechanism of blood protein uptake by insect oocytes, Am. Zool., 3, 185-191.

Thomson, E. and Noller, I. (1963), Influence of neurosecretory cells and corpus allatum on intestinal proteinase activity in the adult *Calliphora erythocephala* Meig., J. Exp. Biol., 40, 301-322.

Timmins, W.A.and Reynolds, S.E. (1992), Azadirachtin inhibits secretion of trypsin in midgut of *Manduca sexta* catapillars: reduced growth due to impaired protein digestion, Entomologia Exp. Appl., 63, 47-54.

Trimen, H. (1974), A handbook to the Flora of Ceylon, Part I, Bishen Singh & Periodical Experts, Delhi, 327.

Turner, A.E., and Loughton, B.G. (1975) *In vitro* protein synthesis by tissues of the fifth instar locust, Insect Biochem. 5, 791-804.

Turner, C. J. ; Tempesta, M. S. ; Taylor, R. B.; Zagorski, M. G.; Termini, J.S.; Schroeder, D. R. and Nakanishi, K. (1987) Analysis of NMR spectroscopic study of azadirachtin and its trimethyl ether, Tetrahedron, 43, 2789-2803.

Uebel, E.C., Warthen, J.D. and Jacobson, M. (1979) Preparative reversed phase liquid chromatographic isolation of azadirachtin from neem seed kernals , J. liq. chro., 2, 875-882.

Van Beek T.A. & Ae de Groot, (1986), Terpenoid antifeedants part 1. An overview of terpenoid antifeedants of natural origin, Recl. Trav. Chim. Pays-Bas 105,513-527.

Van der Horst, D.J., Van Doorn, J.M., Pener, M.P., Van den Broek, A.T.M., Van Marrewijk, W.J.A. and Beenakkers, A.M.T. (1989), Development of imaginal competence to adipokinetic hormone in *Locusta* : lipophrin conversions in precocene-induced adultiforms and in azadirachtin-induced over-aged nymphs, Comp. Biochem. Physiol., 92B, 133-136.

Vanderberg, J.P. (1963) Synthesis and transfer of DNA, RNA and protein vitellogenesis in *Rhodnius prolixus*, Biol. Bull., Wood Hole, 125, 556-575.

Verma, S.P., Singh, B. and Singh, Y.P. (1985), Studies on the comparative efficacy of certain grain protectants against, *Sitotroga cerealella* Oliver., Bull. Grain Tech., 24,37-42.

Verma, V.S. (1974), Chemical compounds from *Azadiracha indica* as inhibitors of potato virus, Acta Mocrobiologica Polanica, 6B, 9-13.

Vijayalakshmi, K., Gaur, H.S. and Goswami, B.K. (1985), Neem for the control of plant paracitic menatodes, Neem Newsl., 2, 35-42.

Warthen, J.D., (1989), Neem (*Azadirachta indica* A. Juss): Organisms affected and reference list update, Proc. Entoml. soc. Wash., 91, 367-388.

Warthen, J.D.; Stokes, J.B.; Jacobson, M. and Kozempel, M.F. (1984) Estimation of azadirachtin content in neem extract and formulations, J. liq. chroma., 7, 591-598.

Warthen, J.H.Jr. (1979), *Azadirachta indica,* a source of insect feeding inhibitors and growth regulators. USDA Sci. & Educ. Adm. Agric. Reviews and Manuals North-eastern Ser. 4, 1-21.

West, A.J. and Mordue, A.J. (1992), the influence of azadirachtin on the feeding behaviour of cereal aphids and slugs, Entomologia exp. appl., 62, 75-79.

Whitton, P.S. (1987), Stress induced traurine release in insects and effect on the central nervious system, Ph.D. thesis, University of Glasgow, Glasgow.

William, R.J., Jr. Lansford, E.M. (Eds), (1967), The encyclopedia of biochemistry, Reinhold publishing corporation, London, 684.

Wilps, H., Kirkilionis, E. and Muschenich, K. (1992), The effect of neem oil and azadirachtin on mortality, flight activity, and energy metabolism of *Schstocerca gregaria* (Forskal) - a comparision between laboratory and field locusts, Comp. Biochem. Physiol., 102c, 67-71.

Wyatt, G.R. (1988) Vitellogenin synthesis and the analysis of juvenile action in locust fat body, Can. J. Zool., 66, 2600-2610.

Wyatt, G.R.; Chen, T.T.; and Couble, P. (1976) Juvenile hormone induced vitellogenin synthesis in locust fat body *in vitro* . In " Inveribrate Tissue culture- Application in Medicine, Biology, and Agriculture: Eds: E. Kurstak and K. Maramorosch, 195-202, Academic press, New york.

Yamasaki, R.B. & Klocke, J.A. (1987) Structure bio-activity relationships of azadirachtin, a potent insect control agent, J. Agric. Food Chem., 35,467-471.

Yamasaki, R.B.; Klocke, J.A.;Lee, M.Z.; Stone,G.A, and Darlington,M.V. (1986) Isolation and purification of azadirachtin from neem *(Azadirachta indica)* seeds using flash chromatography and high performance liquid chromatography, J. Chromatography, 356,220-226.

Zanno, P.R.; Muira, E.; Nakanishi, K. and Elder, D.L. (1975) Structure of the insect phagorepellant azadirachtin , Application of PRFT/CWD carbon-13 nuclear resonance, J. Amer. Chem. Soc. 97, 1975-1977.

Zongza, V. and Dimitriadis, G.C. (1988) vitellogenesis in the insect *Dacus oleae*. Isolation and characterization of yolk protein mRNA, Insect Biochem., 18, 651-660.

APPENDIX

^{1}H-NMR shifts and assignments of salannin and deacetylsalannin

	Salannin	Deacetylsalannin
^{1}H atom	^{1}H chemical shift & Multipicity	^{1}H chemical shift & Multipicity
1	4.97(brs)	5.02(t)
2		2.08(ddd)
3	4.79(br.s)	3.02(s)
5	2.81(d)	2.73(d)
6	3.98(dd)	4.01(dd)
7	4.19(br.s)	4.18(d)
9	2.75(dd)	2.64(dd)
11ab	2.10-2.35(m)	2.12-2.38(m)
& 12ab		
15	5.45(t)	5.39(br.t)
17	3.60(br.d)	
18	1.69(s)	1.64(s)
19	0.98(s)	0.97(s)
21	7.32(br.s)	7.31(s)
22	6.30(br.s)	6.27(br.s)
23	7.27(br.s)	7.25(br.s)
28a	3.70(d)	
28b	3.70(d)	4.13(d)
29	1.22(s)	1.18(s)
30	1.30(s)	1.30(s)
3^1	3.96(q)	6.91(qd)
4^1	1.81(d)	1.85(d)
5^1	1.95(s)	1.92(s)
OAc	1.95(s)	2.42(s)
OMe		2.42(s)
OH	-	2.45(s)

^{13}C-NMR shifts and assignments of salannin and deacetylsalannin

	Salannin	Deacetylsalannin
^{13}C atom	^{13}C chemical shift & Multipicity	^{13}C chemical shift & Multipicity
1	70.5(d)	72.7(d)
2	29.4(t)	30.4(t)
3	67.0(d)	70.7(d)
4	45.4(s)	44.1(s)
5	37.0(d)	38.7(d)
6	74.4(d)	72.4(d)
7	76.4(d)	85.8(d)
8	50.2(s)	48.9(s)
9	44.7(d)	39.4(d)
10	52.5(s)	40.7(s)
11	104.1(s)	30.2(t)
12	171.1(s)	172.5(s)
13	69.9(s)	134.8(s)
14	68.5(s)	146.5(s)
15	73.8(d)	87.8(d)
16	25.1(t)	41.2(t)
17	48.8(d)	49.3(d)
18	20.9(q)	12.9(q)
19	18.4(q)	15.1(q)
20	83.6(s)	127.1(s)
21	107.3(d)	138.7(d)

22	108.7(d)	142.5(d)
23	147.0(d)	142.8(d)
28		77.7(t)
29		19.8(q)
30	72.9(t)	16.8(q)

^{1}H-NMR and ^{13}C-NMR shifts and assignments of azadirachtin H

^{1}H atom	^{1}H chemical shift & multipicity	^{13}C atom	^{13}C chemical shift & multipicity
1	5.3(dd)	1	71.5(d)
2b	2.3(ddd)	2	28.9(t)
3	5.5(dd)	3	66.2(d0
5	3.3(d)	4	51.4(s)
6	4.46dd)	5	35.8(d)
7	4.6(d)	6	73.2(d)
9	3.1(d)	7	75.0(d)
11	5.41(d)	8	42.7(s)
15	4.5(d)	9	46.9(d)
16a	1.7(ddd)	10	47.3(s)
16b	1.3(d)	11	99.5(d)
17	2.3(d)	12	-
18	1.9(s)	13	66.6(s)
19a	3.7(d)	14	69.3(s)
19b	4.0(d)	15	76.0(d)
21	5.6(s)	16	24.7(t)
22	5.0(d)	17	47.1(d)
23	6.4(d)	18	17.7(q)
28a	4.0(d)	19	68.1(t)
28b	3.7(d)	20	81.4(s)

30	1.6(s)	21	107.5(d)
7-OH	1.6(s)	22	107.0(d)
11-OH	2.6(s)	23	144.8(d)
20-OH	2.9(s)	28	71.5(t)
29-OMe	3.7(s)	29	172.9(s)
OAc	1.8(s)	30	19.9(q)
3^1	6.9(qq)	COOMe	51.3(q)
4^1	1.7(dq)	COOMe	-
5^1	1.8(dq)	OAc	168.8(s)
		OAc	19.6(q)
		1^1	165.2(s)
		2^1	127.4(s)
		3^1	136.9(d)
		4^1	13.4(q)
		5^1	10.9(q)

Table-1 shows the composition of the culture medium.

Components	amount mg/l
Inorgainc salts	
$CaCl_2$	980
KCl	2900
$MgCl_2$	1070
$MgSO_4$	1370
$NaHCO_3$	350
$NaH_2PO_4.H_2O$	1100
Amino acids	
L-alanine	225
L-arginine	550
L-asparagine	350

L-aspartic acid	350
L-cystine	20
L-glutamic acid	600
L-glutamine	600
Glycine	650
L-histidine	3400
L-isoleucine	50
Leucine	75
L-lycine. HCl	630
L-methionine	50
L-phenylalanine	150
L-proline	350
L-serine	550
L-threonine	350
L-tryptophan	100
L-tyrosine	55
L-valine	100
Other components	
D-glucose	1000
Tryptose broth	2600